IMPERIAL AGRICULTURAL
SEARCH INSTITUTE, NEW DELHI.

PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

WARDER C. ALLEE
Managing Editor

EDITORIAL BOARD

J. H. BODINE, University of Iowa
C. M. CHILD, Stanford University
M. F. GUYER, University of Wisconsin
L. V. HEILBRUNN, University of Pennsylvania
E. E. JUST, Howard University
F. R. LILLIE, University of Chicago
R. S. LILLIE, University of Chicago
W. W. SWINGLE, Princeton University
C. V. TAYLOR, Stanford University
B. H. WILLIER, University of Rochester

VOLUME XI
1938

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO ILLINOIS

THE CAMBRIDGE UNIVERSITY PRESS, LONDON
THE MARUZEN COMPANY, LIMITED, TOKYO
THE COMMERCIAL PRESS, LIMITED, SHANGHAI

PUBLISHED
JANUARY, APRIL, JULY, OCTOBER, 1938

COMPOSED AND PRINTED BY THE UNIVERSITY OF CHICAGO
PRESS, CHICAGO, ILLINOIS, U.S.A.

CONTENTS OF VOLUME XI

NUMBER 1

The Relation between Temperature, Salts, Hydrogen Ion Concentration, and Frequency of Ingestion of Food by *Amoeba* . *S. O. Mast and R. A. Fennell* 1

The Effect of Chemicals and Temperature on Reversal in Ciliary Action in *Paramecium* *Joseph F. Oliphant* 19

The Influence of Certain Plant Hormones on Growth of Protozoa . *Alfred M. Elliott* 31

Some Effects of Hypertonic Solutions upon Development and Oxygen Consumption *Arthur Louis Schipper* 40

A Study of the Mechanism of Cyanide Inhibition. I. Effect of Concentration on the Egg of *Melanoplus differentialis* . . *W. A. Robbie, E. J. Boell, and J. H. Bodine* 54

Comparison of Quantitative Precipitin Techniques as Influenced by Injection Procedures *Harold R. Wolfe and Joseph G. Baier, Jr.* 63

The Influence of the Frequency of Stimulation on the Slow and the Fast Contraction in Crustacean Muscle . . . . *C. A. G. Wiersma and A. Van Harreveld* 75

The Response to Mechanical Shock by the Cercariae of *Bucephalus elegans* *Harry T. Folger and Lloyd E. Alexander* 82

The Nutritional Requirements of *Blattela germanica* . . *C. M. McCay* 89

NUMBER 2

Annotated Bibliography of the Scientific Publications of Professor Charles Manning Child *Libbie H. Hyman and C. D. Van Cleave* 105

The Fragmentation of *Nais paraguayensis* . . . . *Libbie H. Hyman* 126

The Effect of Planarian Extracts and Exudates upon Head Regeneration in *Euplanaria dorotocephala* . . . . *J. William Buchanan* 144

The Microscopic Anatomy of Twins and Double Monsters of *Fundulus heteroclitus* *Marie A. Hinrichs* 155

The Influence of Thyroxin on Eye Formation in *Phagocata gracilis* (Haldeman) *Bernal R. Weimer, Charles W. Phillips, and Dagney M. Andersen* 158

The Effect of Dead Optic Vesicle upon Explants of Prospective Ectoderm *C. D. Van Cleave* 168

Oxygen as a Controlling Factor in the Regeneration of Tubularia . . . *L. G. Barth* 179

Differential Gradient Functions in the Feather Germ . . . . *Richard M. Fraps* 187

Single and Combined Effects of Cyanide and Methylene Blue on Reconstitution in *Euplanaria dorotocephala* . . . . *Olin Rulon* 202

Studies on Heteroplastic Transplantation in Triclads. I. Cephalic Grafts between *Euplanaria dorotocephala* and *E. tigrina* . . . . *James A. Miller* 214

NUMBER 3

Modifications of Development in Relation to Differential Susceptibility of the Bruchid (Coleoptera) Egg to KCN during Different Metabolic Phases . . . *Alfred Brauer* 249

Ultra-Violet Radiation of Grasshopper Eggs (Orthoptera) *O. Malcolm Ray* and *Joseph Hall Bodine* 267

The Rate of Oxygen Consumption of Frogs during Embryonic Development and Growth *Meyer Atlas* 278

Light Intensity and Melanophore Response in a Cyprinid Fish *Ragnar Nathanael Danielson* 292

The Similarity between the Hypophyseal Chromatophorotropic Hormone and the Chromatophorotropic Hormone of the Crustacean Eyestalk . *A. A. Abramowitz* 299

On the Motor and Inhibitory Innervation of the Legs of Brachyuran Decapods *C. A. G. Wiersma and George Marmont* 312

The Olfactory Reactions of *Drosophila melanogaster* Meigen to the Products of Fermenting Banana . . . . *Melia R. Reed* 317

Race and Conjugation of *Paramecium* . . . . *Arthur C. Giese* 326

Reproduction of *Paramecium* in Old Culture Medium *W. H. Johnson and Garrett Hardin* 333

Studies on the Physiology of Amoeba. II. The Allelocatalytic Effect in Amoeba Cultures Free of Bacteria . . . . *Karl Reich* 347

NUMBER 4

Erratum . . . .

The Effect of Substances Produced by *Chilomonas paramecium* on Rate of Reproduction *S. O. Mast and D. M. Pace* 359

Fat Release in Amoeba after Irradiation . *L. V. Heilbrunn and Kathryn Daugherty* 383

An Experimental Study of Clot Formation in the Perivisceral Fluid of *Arbacia* *James A. Donnellon* 389

Abundant Feeding Followed by Restricted Feeding and Longevity in *Daphnia* *H. Howard Dunham* 399

Studies in Mass Physiology: Effect of Numbers upon the Oxygen Consumption and Locomotor Activity of *Carassius auratus* . . . . *Arthur Shlaifer* 408

Fetal Respiratory Movements in the Cat *W. F. Windle, M. Monnier, and A. G. Steele* 425

Physiology of Development of the Feather. II. General Principles of Development with Special Reference to the After-Feather . . . . *Frank R. Lillie and Mary Juhn* 434

Index to Volume XI 451

ERRATUM

In the third paragraph of XI, No. 3 (July), 320, the first sentence should read: "To test the possibility of escape, a hundred flies, weighing about 41.1 mg. per hundred, were counted into each of three traps, and a hundred large flies, weighing about 124.1 mg. per hundred, were counted into each of three other traps."

Physiological Zoölogy

Vol. XI JANUARY 1938

THE RELATION BETWEEN TEMPERATURE, SALTS, HYDROGEN ION CONCENTRATION, AND FREQUENCY OF INGESTION OF FOOD BY AMOEBA[1]

(Eight figures)

S. O. MAST AND R. A. FENNELL
Zoological Laboratory, Johns Hopkins University

INTRODUCTION

IT IS well known that under some conditions amoebae do not feed, although the culture medium contains an abundance of food, and that under other conditions some feed and others do not. This shows that feeding in amoebae depends upon the condition of the organisms and the condition of the environment. However, only a few detailed observations have been made concerning the determining factors.

Schaeffer (1916) maintains that the most important single factor in inducing feeding in amoebae is movement of the food, but that the character of the food particles taken in is also involved. He says that, in general, soluble particles are more readily taken than insoluble ones.

Mast and Hahnert (1935) came to the following conclusion:

> *Amoeba proteus* does not feed when it is not attached to the substratum, therefore anything which facilitates attachment facilitates feeding. It has been repeatedly observed that feeding increased greatly if amoebae were transferred from a solution in which attachment was weak, to one in which it was strong, e g., fresh Chalkley solution. It was repeatedly observed that feeding increased if the amoebae were agitated. This was particularly marked in specimens transferred from ordinary culture dishes to slides. Starvation facilitates feeding, but not if it continues until the amoebae no longer attach firmly. Specimens kept without food 5 to 8 days usually do not feed again no matter how they are treated, although they live 10 days or longer.

Aside from these facts, little is known concerning the factors which determine feeding in *Amoeba*. More is known, however, concerning these factors in other protozoa.

Schaeffer (1910) made a very thorough study of feeding in *Stentor caeruleus*. He maintains that hunger accelerates frequency of ingestion, that satiety retards it, and that *Stentor* takes some organisms more readily than others.

---

[1] We are indebted to the Marine Biological Laboratory at Woods Hole, Massachusetts, for facilities used in making some of the experiments considered below.

Metalnikow (1912) maintains that low temperatures, high temperatures, weak alkaline solutions, and old culture solutions retard frequency of ingestion of particles by paramecia, and that moderate temperatures, weak acid solutions, alcohols, and arsenious compounds accelerate it. He found also that paramecia ingests digestible substances more readily than indigestible substances.

MATERIALS AND METHODS

Kaulbaum's "purest" chemicals and triple (Pyrex glass) distilled water were used in all solutions. The salts were carefully dried and weighed, and in some instances purified by recrystallization. The concentration of the hydroxide solutions used was ascertained by titrating against a known amount of potassium acid phthalate ($KHC_8H_4O_4$), as outlined by Clark (1928). The solutions were stored in Pyrex glass flasks, which contained an outlet with a stopcock and an inlet with a soda-lime tube attached. The concentration of $MgCl_2$ and $CaCl_2$ was ascertained by the method described by Blasdale (1931). The concentration of the phosphates used was ascertained by the molybdenum-blue method of Deniges, as modified by Farber and Youngburg (1932). Calcium chloride was substituted for $CaH_4(PO_4)_2$ in solutions with low hydrogen ion concentrations, so as to avoid precipitation of the calcium.

The temperature of the solutions in which feeding was tested was controlled by means of a temperature bath, designed by Pitts (1932). This bath consists of a small brass box which fits on the stage of the microscope and contains a flat-bottom glass dish surrounded by a rubber diaphragm to prevent leakage of water. The glass dish was made as follows: A 50-cc. Pyrex glass beaker was painted inside and outside with paraffin; then a line in the paraffin 2 mm. wide was made around the beaker 2.5 cm. from the bottom, after which it was immersed in hydrofluoric acid and left 24 hours. During this time the acid dissolved the glass which was not covered by paraffin, separating the bottom from the top. The rough edges of the bottom were ground down and polished. The temperature in the glass dish was adjusted by regulating the flow of water of the desired temperature through the bath. The hydrogen ion concentration of the solutions was measured electrically.

Chilomonas paramecium cultured in sterile glucose-peptone solution (1.5 g. glucose and 3.5 g. peptone in 1,000 cc. water) was used as food in all experiments made. Sterile cultures were obtained by a method similar to that which was perfected by Hargitt and Fray (1917) and Phillips (1922). Twenty-five chilomonads were put into 0.4 cc. of glucose-peptone solution on a sterile depression slide in a sterile Petri dish. Then the dish was covered and left 5 minutes. The chilomonads were then transferred with a sterile pipet (plugged with sterile cotton at the bulb end) to the same amount of solution on another depression slide in another Petri dish, which was covered and left 5 minutes. This process was then repeated thirteen times, after which one of the chilomonads was transferred to each of fourteen sterile 125 cc. flasks, each of which contained 50 cc. of sterile glucose-peptone solution.

The flasks were left from 5 to 7 days; then one of the sterile flasks was used to inoculate additional flasks, each containing 50 cc. of sterile glucose-peptone solution. The freshly inoculated flasks were left 48 hours, that is, until chilomonads were abundant. Then the chilomonads in three of these flasks were transferred, as follows, to the solution used in the tests on feeding: Fifteen cubic centimeters of the solution in one of the flasks was put into each of two centrifuge tubes and centrifuged at a moderate speed for

5 minutes, after which all but 1 cc. of the solution (i.e., the solution which contained the chilomonads) was poured off. Then 14 cc. of the solution from the flask was added to each test tube. This was repeated until all the solution in the three flasks was centrifuged. Then 14 cc. of the solution to be used for the test on feeding was added to each tube and centrifuged, after which all but 1 cc. at the bottom of the tube was poured off. This procedure was repeated three times. During the successive transfers the glucose-peptone in which chilomonads were cultured was diluted about 40,000 times. Chilomonads were prepared in this way for each test on feeding, as described below.

The amoebae used were cultured in Chalkley solution (see p. 4) containing rice and wheat. Three weeks after inoculation the best culture was selected; and from this 250 amoebae[2] of the same size and appearance were taken, put into a small Pyrex glass dish which contained 5 cc. distilled water, and left 10 minutes. Then they were transferred through the same amount of distilled water three more times, but after the last transfer they were left for 24 hours instead of 10 minutes. At this time each amoeba had three or four long, slender pseudopods and little, if any, food (no fat or starch), and most of them were not attached or only loosely attached to the substratum. From these 250 amoebae, 100 as nearly as possible the same in size, structure, and form were selected and put into a small beaker which contained 5 cc. of the solution to be used in the test on feeding, and left 5 minutes. Then they were transferred, in the same way, through the same amount of solution three more times, after which they were transferred to the same amount of solution in the small glass dish in the Pitts temperature bath, which was now covered, sealed with vaseline, and left undisturbed for 15 minutes for adjustment to the solution. Then the cover of the dish was removed, and 2 cc. of the solution containing chilomonads prepared for tests on feeding were added, after which the dish was again covered and sealed. Fifteen minutes after adding the chilomonads to the bath, 10 amoebae were taken out, put on a glass slide between parallel ridges of vaseline, and covered with a cover slip. Then some of the solution was removed with filter paper so as to flatten the amoebae; then the chilomonads in each amoeba were counted, and the mean calculated and recorded. This was repeated 30, 60, and 120 minutes after the chilomonads had been in the Pitts bath. The results obtained are considered in the following pages.

THE RELATION BETWEEN ABUNDANCE OF FOOD AND FREQUENCY OF INGESTION BY *Amoeba proteus*

Metalnikow (1912) maintains that in paramecia the rate of feeding is, within wide limits, independent of the abundance of food in the culture solution; but Fenn (1921) maintains that in leucocytes the number of particles ingested by them is directly proportional to the number of particles in suspension.

One hundred selected amoebae in Chalkley solution were put into each of two Pitts temperature baths, adjusted to 24° C. Then about 925,000 chilomonads were added to one and 1,850,000 to the other, after which Chalkley solution was added until the volume in both was 3 cc.; then the number of chilomonads ingested by the amoebae was ascertained, as described above.

The results are given in Table 1. These results show that the number of chilomonads ingested in 15- and 30-minute periods was slightly higher in the solution which contains the greater number of chilomonads, but that the number ingested in 60 and 120 minutes was nearly the same in both solutions. This demonstrates that there can be great variation in the abundance of chilomonads in the solution without appreciably affecting the number of chilomonads ingested by the amoebae, if they are left half an hour or longer.

---

[2] Each time that amoebae were removed from a culture, the solution taken out with them was replaced with distilled water. Owing to this, the hydrogen ion concentration in the cultures remained nearly constant.

THE RELATION BETWEEN TEMPERATURE AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus and Amoeba dubia*

Observations were made on ingestion of chilomonads by *A. proteus* in Chalkley solution,[3] and by *A. dubia* in Hahnert (1932) solution, at temperatures of 5°, 10°, 15°, 20°, 25°, 30°, and 35° C. These observations were made as described in the preceding pages.

TABLE 1

THE RELATION BETWEEN ABUNDANCE OF FOOD AND FREQUENCY OF INGESTION BY *Amoeba proteus* (6 TO 10 AMOEBAE USED IN EACH TEST)

NUMBER OF CHILOMONADS PER CUBIC CENTIMETER	AVERAGE NUMBER OF CHILOMONADS INGESTED BY 6–10 AMOEBAE				NUMBERS INGESTED PER INDIVIDUAL IN 120 MIN.	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
600,000....................	12	41	56	80	63	96
300,000....................	9	36	57	81	64	98

Three sets of observations were made, two on *A. proteus* and one on *A. dubia*. The results obtained in the first set of observations on *A. proteus* and those obtained in observations on *A. dubia* are presented in Table 2 and Figure 1. Figure 1 shows, in these two sets of observations, that, as the temperature increased, the frequency of ingestion of chilomonads by *A. proteus* and by *A. dubia* increased to a maximum at about 26° and then decreased to zero at 40°, but that the number ingested by *A. proteus* in 120 minutes decreased to a median minimum at 30°, and to a second maximum at 35°, before it decreased to zero. Similar results were obtained in the second set of observations on *A. proteus*, but there was no median minimum. The median minimum obtained in the first set is therefore probably not significant. The results as a whole, show that frequency of ingestion in amoebae is closely correlated with temperature.

Table 2 shows that at 25° an average of 63 chilomonads per amoeba were ingested in 120 minutes and that, of these, 26 were ingested during the first 15 minutes, 10 during the next 15 minutes, 20 during the next 30 minutes, and only 7 during the last 60 minutes. The frequency of ingestion therefore decreased from 1.73 chilomonads per minute during the first 15 minutes, when the amoebae contained from 0 to 25 chilomonads each, to only 0.1 chilomonad per minute during the last 60 minutes, when they contained from 56 to 63 each. Table 2 shows also that similar results were obtained at 5°, 10°, 15°, 20°, 25°, and 30° C. Similar results were also obtained in the second set of tests with *A. proteus* and in the set of tests with *A. dubia*. It is consequently evident that the frequency of ingestion varies inversely with the amount of food in the amoebae.

---

[3] Chalkley solution: 80 mg. NaCl, 4 mg. $NaHCO_3$, 4 mg. KCl, 4 mg. $CaCl_2$, 2 mg. CaH_4 $(PO_4)_2$, 2 mg. $Mg_3(PO_4)_2$ $4H_2O$, and 1,000 cc. distilled water. Hahnert solution: 4 mg. $NaHCO_3$, 4 mg. KCl, 4 mg $CaCl_2$, 2 mg. $CaH_4(PO_4)_2$, 2 mg. $Mg_3(PO_4)_24H_2O$, and 1,000 cc. distilled water.

TABLE 2

THE RELATION BETWEEN TEMPERATURE AND THE FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus*

Temperature in Degrees C.	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers Ingested per Individual in 120 Min.	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
5	0 8	0 6	6	9	4	16
10	1 1	7	12	13	6	25
15	2	10	18	43	32	56
20	7	19	37	50	38	60
25	26	36	56	63	46	72
30	25	43	50	51	42	65
35	13	10	43	68	46	86
40	0	0	0	0	0	0

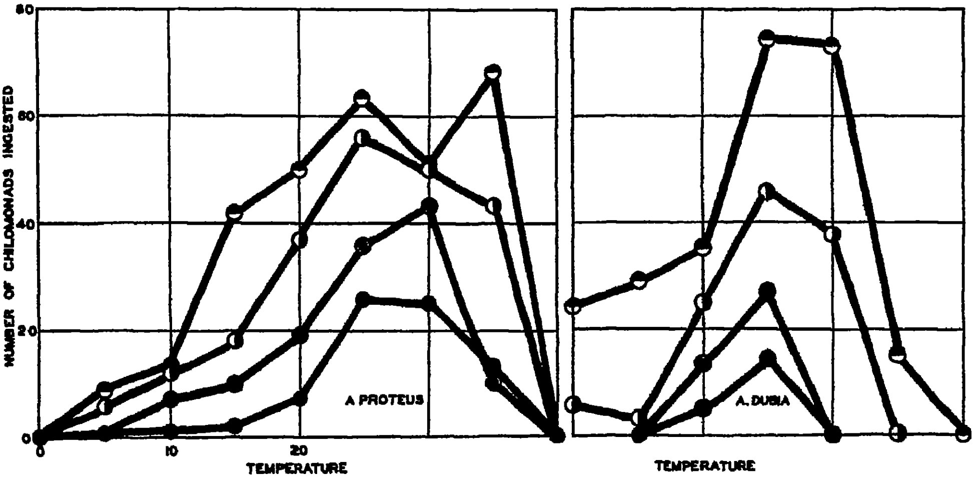


FIG. 1.—The relation between temperaure and frequency of ingestion of chilomonads by *Amoeba*. ●, number of chilomonads ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals. The extent of individual variation in *A. proteus* is presented in Table 2. The variation in *A. dubia* was practically the same.

Table 2 shows that at 35° an average of 13 chilomonads were ingested during the first 15 minutes, none during the next 15 minutes, 33 during the next 30 minutes, and 25 during the last 60 minutes, i.e., that the frequency of ingestion decreased from 0.86 chilomonads per minute during the first 15 minutes to zero during the second, then increased to 1.10 during the next 30 minutes, and then decreased to 0.41 during the last 60 minutes. These results indicate that the frequency of ingestion at 35° is not closely correlated with the number of chilomonads in the amoebae. This is probably due to variations in attachment to the substratum, for the amoebae were fairly firmly attached during the first 15 minutes, when the frequency of ingestion was high, and only loosely during the second 15 minutes, when it was low, but were again fairly firmly attached after that, when it was again high. This indicates that the frequency of ingestion varies directly with the degree of attachment to the substratum, and that the increase in frequency after exposure of 30 minutes at 35° is due to adjustment, resulting in increase in the degree of attachment.

Mast and Prosser (1932) maintain that, as the temperature increases, the rate of locomotion in *A. proteus* increases to a maximum at about 25°, then decreases to a median minimum at about 28°, then increases to a second maximum at about 30°, and then rapidly decreases to zero. Schwitalla (1925) obtained results in accord with these views. This indicates that rate of locomotion is correlated with temperature in much the same way as frequency of ingestion.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING SODIUM AS THE ONLY METALLIC CATION AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The experiments described in this section were made with two series of solutions which consisted of NaH_2PO_4 and NaOH in water. Each of the two series was prepared by mixing NaH_2PO_4 of a given molecular concentration with NaOH of the same molecular concentration in such proportions as to produce a series of hydrogen ion concentrations from pH 4.4 to 8.3. The concentration of the sodium was consequently the same in all the solutions in each of the two series. It was 0.002 N in one and 0.005 N in the other. To obtain hydrogen ion concentrations of pH 4.4, a small amount of 0.1 M HCl was added. Observations were made as described in the preceding pages. The results obtained are presented in Table 3 and Figure 2.

Figure 2 shows that, as the hydrogen ion concentration decreased, the frequency of ingestion of chilomonads by amoebae increased from zero at pH 4.4, nearly to a secondary maximum at pH 5.0, then remained nearly constant to pH 5.5, then increased to a primary maximum between pH 6.0 and 6.5, then decreased to zero at pH 7.7–8.3. It shows that the rate of ingestion in the stronger solution of sodium was consistently much higher than in the weaker solution and that there was somewhat of a median minimum near pH 6.5 in both concentrations.

Figure 2 indicates that frequency of ingestion is more closely correlated with the hydrogen ion concentration in the stronger than in the weaker solution, and that the maximum acidity at which ingestion occurred was the same in both concentrations but that the maximum alkalinity[4] at which it occurred was higher in the weaker than in the stronger solution.

Table 3 columns 6 and 7, show that there was marked individual variation in the frequency of ingestion in the weaker solution but that the averages are consistent throughout. The variation in the stronger solution was essentially the same as in the weaker.

---

[4] "Maximum alkalinity" is defined as the lowest hydrogen ion concentration in which ingestion occurs; and "maximum acidity," as the highest in which it occurs.

TABLE 3

THE RELATION BETWEEN FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus* AND HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING SODIUM AS THE ONLY METALLIC CATION (CONCENTRATION OF SODIUM, 0.002 N)

Hydrogen Ion Concentration in pH Units	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers Ingested per Individual in 120 Minutes	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
4.4	0	0	0	0	0	0
4.7	16	31	34	43	32	56
5.0	22	34	37	53	39	68
5.5	21	26	36	52	35	74
6.0	23	34	44	51	23	78
6.3	20	29	47	53	35	72
6.6	18	22	33	67	53	76
7.0	19	23	40	47	40	58
7.4	21	38	49	49	20	110
7.8	18	29	42	50	28	70
8.1	17	19	32	44	28	65
8.4	0	0	0	0	0	0

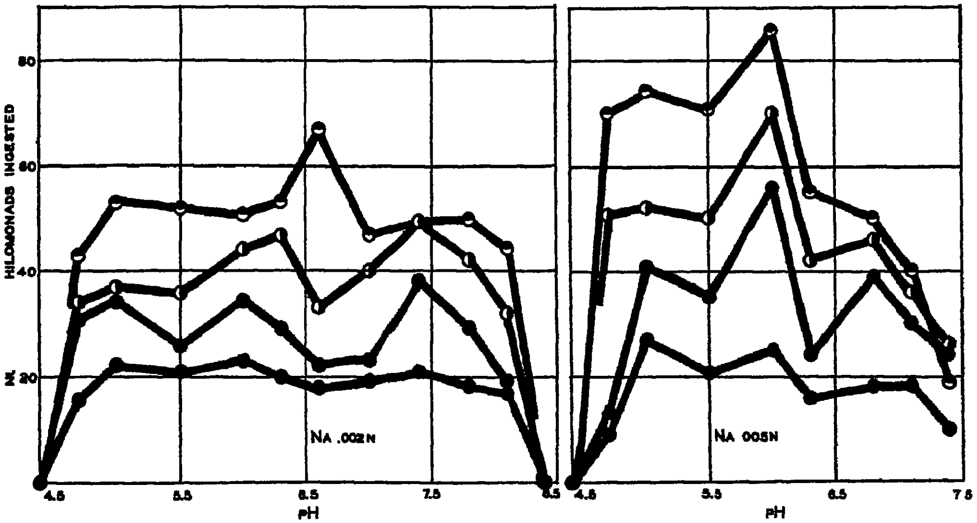


FIG. 2.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing sodium as the only metallic cation. ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◒, 120 minutes. Each point on the curves represents the average for 10 individuals. Lowest and highest numbers of chilomonads ingested per individual in 120 minutes is 32 and 56, respectively, at pH 4.7; 53 and 76 at pH 6.6, the optimum hydrogen ion concentration. Similar individual variations occurred in all the other tests.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING POTASSIUM AS THE ONLY METALLIC CATION AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The methods used in making observations on the effect of potassium on frequency of ingestion of food by amoebae were the same as those used in observations on the effect of sodium. The results are presented in Figure 3.

Figure 3 shows that as the hydrogen ion concentration increased the frequency of ingestion of chilomonads increased from zero at pH 4.4 to a maximum at pH 5.0 and then decreased nearly to zero, but that it decreased much more gradually in the weaker than in the stronger solution and that the maximum alkalinity was considerably higher in the former than in the latter. It indicates that frequency of ingestion of food is more closely correlated with hydrogen ion concentration in the stronger than in the weaker solutions of potassium, as it is in solutions of sodium.

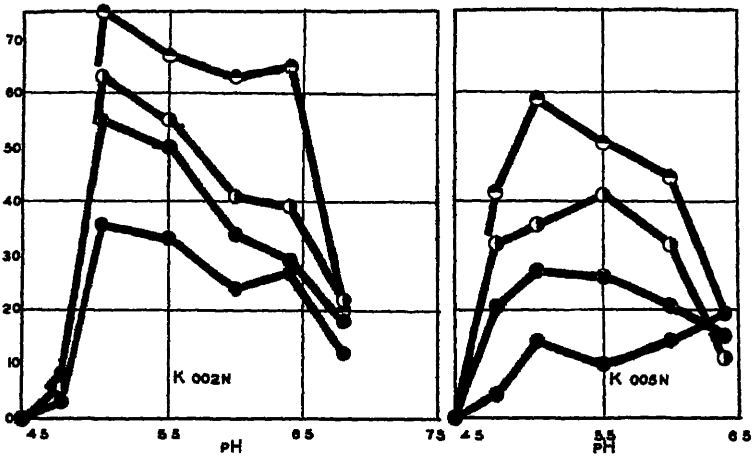


FIG. 3.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing potassium as the only metallic cation ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals. The individual variation was similar to that in solutions containing sodium in place of potassium.

It will be seen by comparing Figure 3 with Figure 2 that the results obtained with potassium are similar to those obtained with sodium—that in both, the alkaline limit for ingestion is more acid in the stronger than in the weaker solution, but that at the optimum for frequency of ingestion, the solutions which contain potassium are more acid than those which contain sodium, and that, as the concentration increased, frequency of ingestion decreased in the former and increased in the latter.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING CALCIUM AS THE ONLY METALLIC CATION AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The methods used in making observations on the effect of calcium on frequency of ingestion of food by amoebae were the same as those used in observations on the effect of sodium, except that $CaCl_2$ was substituted for $CaH_4(PO_4)_2$ in all solutions with hydrogen ion concentrations lower than pH 6.4, because calcium is precipitated in these solutions if phosphates are present, and that the concentration of calcium in the two series of solutions was 0.001 M and 0.0025 M instead of 0.002 M and 0.005 M, and that the observations (owing to increase in hydrogen ion concentration in the alkaline solutions) were continued for only 30 minutes in place of 120 minutes. The results are presented in Figure 4.

Figure 4 shows that, as the hydrogen ion concentration decreased, the frequency of ingestion of chilomonads gradually increased to a maximum approximately at pH 6.0, then remained constant until pH 7.0 was reached, then decreased until the close of the experiment at pH 8.0 but not to zero.

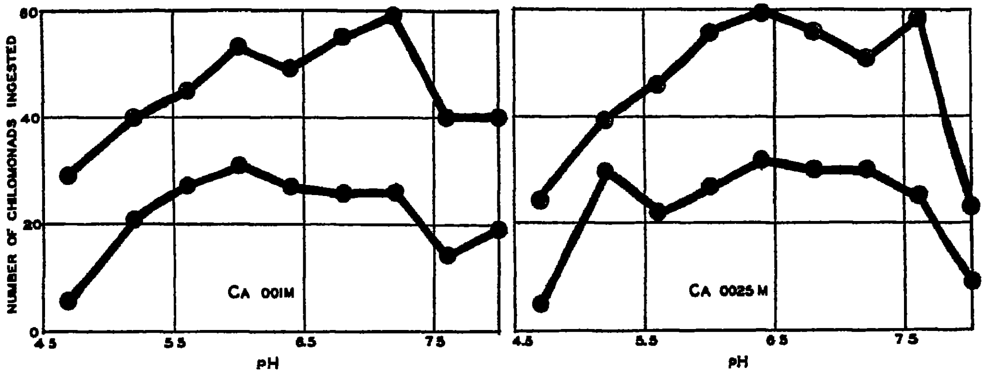


FIG. 4.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing calcium as the only metallic cation. Ordinates, number of chilomonads ingested; abscissae, hydrogen ion concentration in pH units; ●, number ingested in 15 minutes; ◉, 30 minutes. Each point on the curves represents the average for 10 individuals.

Figure 4 also shows that frequency of ingestion of chilomonads was about the same in the weaker and the stronger solutions of calcium, but that the frequency of ingestion at the alkaline limit was much higher in the weaker than in the stronger solution.

It can be seen by comparing Figures 2 and 3 with Figure 4 that the effect of hydrogen ion concentration on frequency of ingestion in calcium solutions differs greatly from that in either sodium or potassium solutions, in that the frequency of ingestion is much higher in the acid and much lower in the alkaline range in the former than in the latter.

The results indicate that the effect of calcium differs qualitatively from either that of sodium or that of potassium. Pitts and Mast (1934) reached the same conclusion in their study on the relations between hydrogen ion concentration in solutions of single salts and rate of locomotion of amoebae.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING SODIUM AND CALCIUM IN DIFFERENT PROPORTIONS AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The observations described in this section were made with two series of solutions which contained NaH_2PO_4, NaOH, and $CaCl_2$ in water. In both series, 0.005 M NaH_2-

PO_4 and 0.005 M NaOH were mixed in various proportions so as to produce solutions which differed in hydrogen ion concentration as described above. To each solution of one series enough $CaCl_2$ was added to make the concentration of calcium 0.001 M; and to each of the other series, enough to make the concentration of calcium 0.003 M. The calcium and the sodium concentrations of all the solutions were therefore the same in each series. The observations were made as described in the preceding pages. The results obtained are presented in Figure 5.

Figure 5 shows that, as the hydrogen ion concentration decreased, frequency of ingestion increased to a maximum at about pH 6.2, then decreased to a median minimum at approximately pH 7, and then decreased to zero at about pH 8.5. The figure shows that the median minimum is much more marked in the solution containing the higher concentration of calcium than that containing the lower concentration, and that the frequency of ingestion, in the alkaline range, was relatively much higher in the former than in the latter.

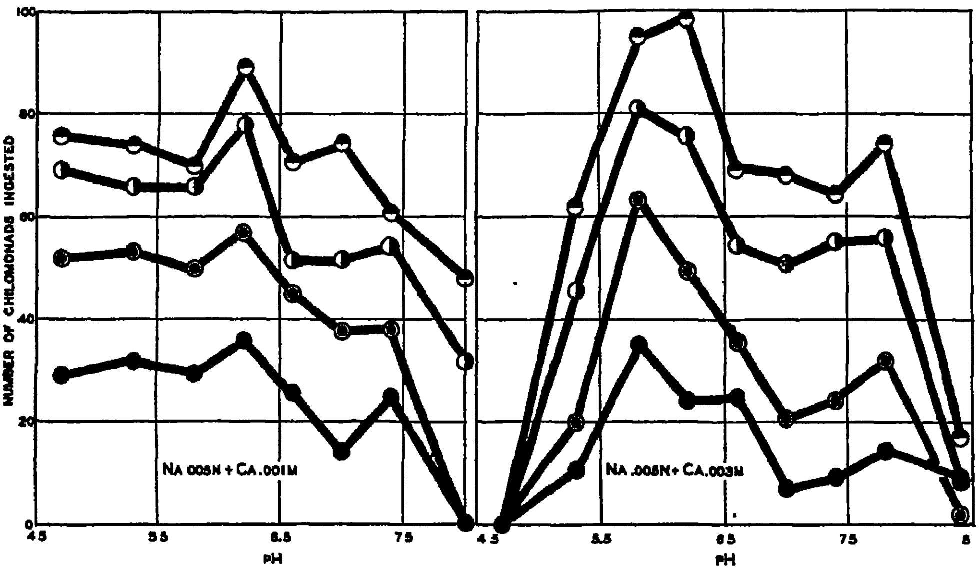


FIG. 5.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing sodium and calcium. *A*, concentration of sodium (0.005 N), calcium (0.001 M); *B*, sodium (0.005 N), calcium (0.003 M); ordinates, numbers of chilomonads ingested; abscissae, hydrogen ion concentration in pH units; ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals.

By comparing Figure 5 with Figure 2, it will be seen that the addition of calcium 0.001 M to solutions containing sodium 0.005 M had little, if any effect on the frequency of ingestion in the stronger acid solutions (pH 4.7–5.7) but that it caused considerable increase in frequency in the weaker acid and the alkaline solutions (pH 6.2–8.0) with a shift in the maximum from pH 6.0 to 6.2, and that addition of calcium 0.003 M caused greater increase in frequency in the weaker acid and the alkaline solutions (pH 5.7–8.2) and marked decrease in the stronger acid solutions (pH 4.7–5.5).

The results obtained in this study are similar to those obtained by Pitts and Mast (1934), who found that, as the hydrogen ion concentration increased, in solutions containing 0.005 N sodium and 0.001 M calcium, rate of locomotion increased from zero at pH 4.5 to a maximum at pH 5.9, then decreased to a median minimum at pH 7.0, then increased to a second maximum at pH 7.5 then, decreased again. Hopkins (1928), using modified Ringer solution, and Mast and Prosser (1932), using a solution containing 0.005 N sodium and 0.002 M calcium, obtained similar results. This supports the conclusion reached in the preceding section, that frequency of ingestion of food and rate of locomotion are closely correlated.

TABLE 4

THE EFFECT OF DIFFERENT CONCENTRATIONS OF NaCl ON FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus*

Molar Concentration of NaCl	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers Ingested per Individual in 120 Minutes	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
0.0003	4	26	35	58	35	73
0.0008	37	72	106	185	154	250
0.002	29	55	93	118	81	163
0.005	29	57	90	110	56	225
0.008	29	45	90	100	62	165
0.01	19	43	86	86	65	120
0.02	25	38	44	55	40	82
0.03	4	7	8	10	0	30

THE RELATION BETWEEN NaCl, KCl, $MgCl_2$, AND $CaCl_2$ IN DIFFERENT CONCENTRATIONS AND THE FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus* and *A. dubia*

The experiments described in this section were made with different concentrations of the chlorides of sodium, potassium, magnesium, and calcium. The observations were made as described in the preceding pages. The results are presented in Table 4 and Figures 6 and 7.

Figure 6 and Table 4 show that, as the concentration of NaCl increased from 0.0003 M, frequency of ingestion of chilomonads very rapidly increased to a maximum at 0.0008 M, then very gradually decreased nearly to zero at 0.03 M, and that at the optimum concentration an average of 185 chilomonads were ingested by each amoeba in 120 minutes.

Figure 6 also shows that the relation between frequency of ingestion and concentration in solutions of KCl is similar to what it is in solutions of NaCl but that the range of concentrations over which ingestion occurs is very much shorter in the former than it is in the latter, and that frequency of ingestion is consistently lower throughout the range, the average maximum frequency being 185 in 120 minutes in NaCl and only 90

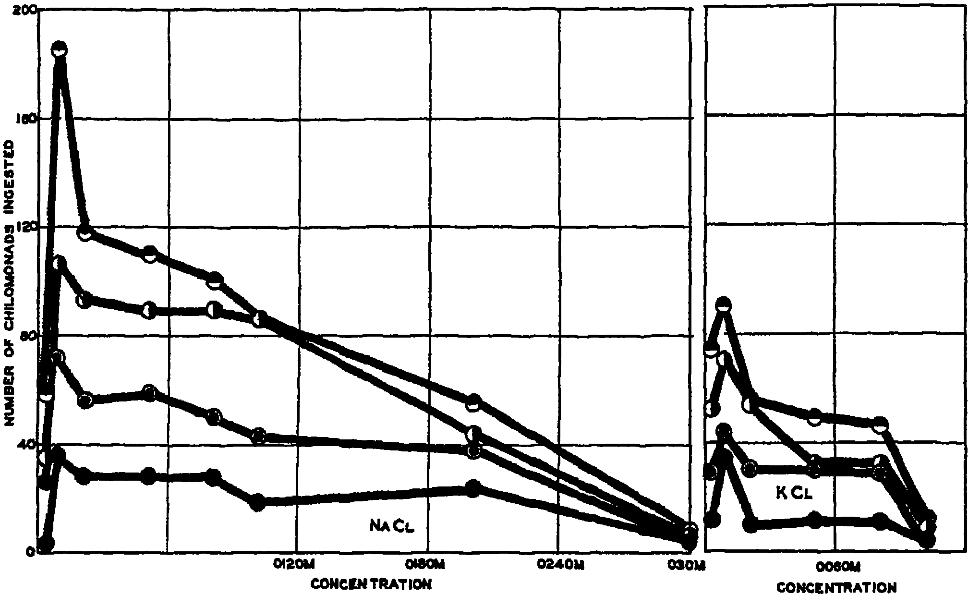


FIG. 6.—The effect of concentrations of salts on frequency of ingestion of chilomonads by *A. proteus*. *A*, NaCl; *B*, KCl; ordinates, number of chilomonads ingested; abscissae, molar concentration; ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals.

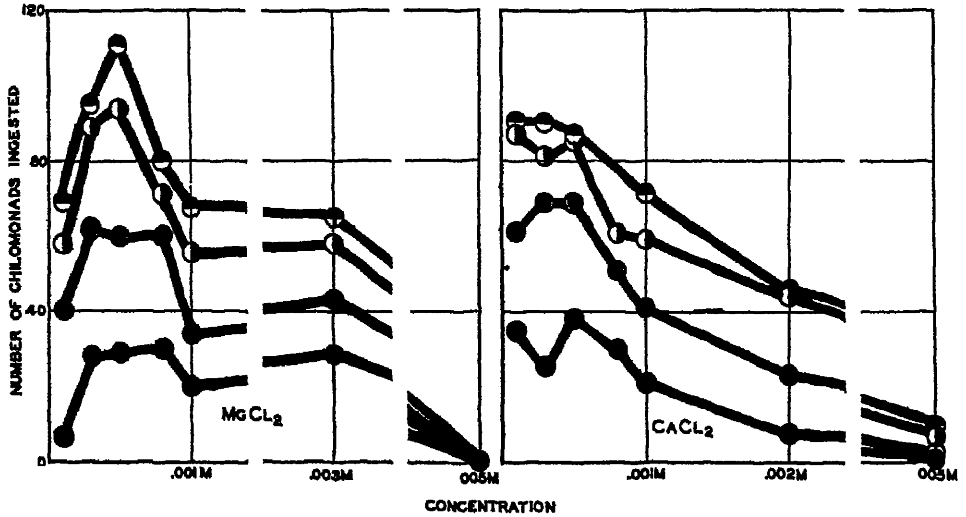


FIG. 7.—The effect of different concentrations of salts on frequency of ingestion of chilomonads by *A. proteus*. *A*, $MgCl_2$; *B*, $CaCl_2$; ordinates, number of chilomonads ingested; abscissae, molar concentration; ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes.

in KCl. The results consequently show that potassium solutions are much less favorable for ingestion than sodium solutions.

These results agree with those obtained by Mast and Prosser (1932) in their study of amoeboid locomotion in different concentrations of KCl. They found that it is extremely difficult to obtain active amoebae in any concentration of KCl, except 0.001 N, and that in this concentration locomotion continued only a short time. Heilbrunn and Daugherty (1932) contend that potassium has strong liquefying action on the plasmagel of amoebae and that it is consequently highly toxic.

Figure 7 shows that, as the concentration of $MgCl_2$ increased from 0.0001 M, frequency of ingestion increased to a maximum at 0.0005 M and then decreased to zero at 0.005 M. It also shows that the relation between frequency of ingestion and concentration in solutions of $CaCl_2$ is similar to what it is in solutions of $MgCl_2$, but that in the lowest concentration used, it is much higher in $CaCl_2$ than in $MgCl_2$ and at optimum concentration somewhat higher in $MgCl_2$ than in $CaCl_2$.

The results presented in Figures 6 and 7 show that the optimum concentration for ingestion of chilomonads by amoebae is at 0.0008 M for NaCl and KCl, at 0.0005 for $MgCl_2$, and probably somewhat lower for $CaCl_2$ The results show also that the maximum frequency of ingestion is highest in NaCl solutions (185), lower in $MgCl_2$ (111), still lower in $CaCl_2$ (91), and lowest in KCl (90); that the maximum concentration in which ingestion occurs is highest in NaCl (0.03 M), much lower in KCl (0.01 M), and still lower in $MgCl_2$ and $CaCl_2$ (0.005 M); and that the minimum concentration in which ingestion occurs is much lower in $MgCl_2$ and $CaCl_2$ (0.0001 M), than in NaCl and KCl (0.0003 M). They show, moreover, that the range of concentration over which ingestion occurs is much greater in NaCl (0.03 M) than in KCl (0.01 M), and much greater in KCl than in $MgCl_2$ and $CaCl_2$ (0.005 M), i.e., that it is more than six times as great in NaCl as in $MgCl_2$ or $CaCl_2$, and about three times as great as in KCl.

The facts that the frequency of ingestion at optimum concentration is approximately twice as high in NaCl as it is in KCl, $MgCl_2$ or $CaCl_2$, and that the range of concentrations over which ingestion occurs is approximately six times as great in NaCl as it is in $MgCl_2$ or $CaCl_2$ and approximately three times as great as it is in KCl, show that ingestion is not specifically correlated with any of the properties common to all these salts—as, for example, osmosis.

THE RELATION BETWEEN OSMOTIC CONCENTRATION AND FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus* AND *A. dubia*

Observations on frequency of ingestion by *A. proteus* and *A. dubia* were made in lactose solution, Chalkley solution (see p. 4), Hahnert solution, and Hahnert solution containing various quantities of lactose. The results are presented in Table 5 and Figure 8.

Table 5 shows that in *A. dubia* the frequency of ingestion was zero in lactose solutions of all concentrations tested, that it was much higher in Chalkley solution than in Hahnert solution, that addition of lactose to Hahnert solution caused marked increase in frequency of ingestion, and that, as the concentration of lactose increases, the frequency increases to a maximum and then decreases.

Figure 8 shows that the results obtained with *A. proteus* are essentially the same as those obtained with *A. dubia*, but that the frequency of ingestion was very much lower, the maximum in 2 hours being more than 400 for the former and only 99 for the latter.

TABLE 5

THE RELATION BETWEEN FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba dubia* AND OSMOTIC PRESSURE

Molar Concentration of Solution Used	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers per Individual Ingested in 120 Minutes	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
0.0001 M lactose	0	0	0	0	....	....
0 0005 M lactose	0	0	0	0	....	....
0 001 M lactose	0	0	0	0	....	....
0 002 M lactose	0	0	0	0	....	....
0 003 M lactose	0	0	0	0	....	....
0 035 M lactose	0	0	0	0	....	....
Chalkley solution	71	137	196	374	358	396
Hahnert solution	34	72	135	193	172	215
Hahnert solution plus 0.03 M lactose	54	123	171	263	240	325
Hahnert solution plus 0.05 M lactose	60	150	232	336	275	405
Hahnert solution plus 0.07 M lactose	51	68	153	169	100	270

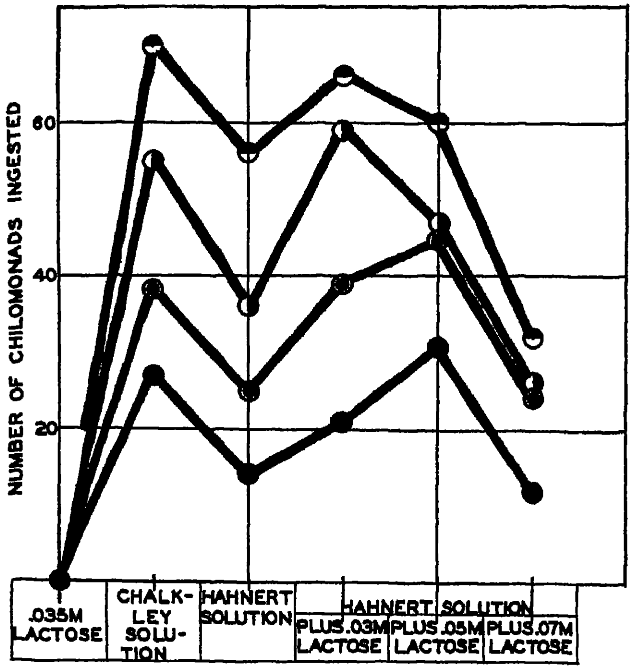


Fig. 8.—The relation between frequency of ingestion of chilomonads by *A. proteus* and osmotic pressure. Ordinates, number of chilomonads ingested; abscissae, molar concentration of solutions; ●, number of chilomonads ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◒, 120 minutes. Each point on the curves represents the average for 10 individuals.

The fact that the frequency of ingestion was higher in Hahnert solution plus lactose than in Hahnert solution indicates that ingestion is correlated with osmotic concentration. The fact that it was zero in lactose solutions and higher in Chalkley solution than in Hahnert solution plus lactose (0.03 M), which was osmotically equal to Chalkley solution, indicates that the effect of salts on ingestion is not entirely due to their osmotic action. The marked difference in the frequency of ingestion in Chalkley and Hahnert solutions is doubtless due to the fact that the former contains NaCl and the latter does not.

DISCUSSION

Mast made a thorough study of the effect of salts, nonelectrolytes, and hydrogen ion concentration on attachment of *A. proteus* to the substratum. He maintains that nonelectrolytes have no effect on attachment, but that all salts greatly facilitate it and that this is not closely correlated with their concentration or the hydrogen ion concentration of the solutions. He says (1929, p. 375):

> All the salts tested (17 in number) greatly decrease the time required for attachment and increase the firmness of attachment. All have the same effect but not in the same concentration
>
> The effect of NaCl and KCl increases very gradually from N/20 to a maximum at about N/100, after which it decreases very gradually until about N/1000 is reached and then very rapidly. The effect of $CaCl_2$ increases rapidly from N/20 to about N/60 then slowly to a maximum at about N/200; after which it decreases very slowly until about N/40,000 is reached and then very rapidly. There is very little difference in the effect on attachment of NaCl and KCl in concentrations ranging from N/40 to N/800 and of $CaCl_2$ in concentrations ranging from N/80 to N/40,000. The effect of $CaCl_2$ is considerably less than that of NaCl and KCl in the stronger and much greater in the weaker solutions tested. All three salts, however, still have a definite effect in the weakest solution tested, N/100,000.
>
> The optimum hydrogen ion concentration for attachment in balanced solution appears to be between pH 6.6 and 7; but there is remarkably little difference over a very wide range, i.e. from pH 4.6 to 8.2.

He found no indication whatever of a median minimum at neutrality.

By comparing these results with those presented in the preceding pages, it is at once evident that, while the effect of nonelectrolytes on attachment seems to be the same as it is on ingestion, the effects of salts and hydrogen ions on ingestion differ greatly from their effects on attachment, and that this is particularly striking in reference to a hydrogen ion concentration of pH 7, in which ingestion was found to be maximum and attachment minimum.

This strongly indicates that, while ingestion is doubtless dependent upon attachment, in accord with the views of Mast and Hahnert (1935), these two phenomena are not closely correlated.

We have demonstrated that the relations between temperature, properties and concentration of salts, and hydrogen ion concentration and frequency of ingestion of food by *Amoeba* are essentially the same as the relation between these environmental factors and the rate of locomotion. This indicates that the factors which control ingestion are fundamentally the same as those which control locomotion.

Pitts and Mast (1934) considered in some detail the mode of action of environmental factors in controlling locomotion. They accounted for some phases of the phenomena involved; but they could give no consistent explanation of the relation between hydrogen ion concentration and rate of locomotion in balanced salt solution, especially the extraordinary reduction in rate at neutrality.

We have no suggestions to offer concerning the mode of action of environmental factors in controlling ingestion except that it probably is essentially the same as it is in reference to locomotion, and we have nothing of value to add to the discussion of Pitts and Mast concerning this.

The results presented demonstrate clearly that in optimum concentration sodium is much more favorable for ingestion than potassium, calcium, or magnesium. We have no suggestions concerning the causes of this.

SUMMARY

1. A quantitative method was devised and used in studying the frequency of ingestion of food by amoebae.

2. Frequency of ingestion varies inversely with the amount of food in the amoebae, but there can be large variations in the abundance of food in the surrounding medium without appreciably affecting frequency of ingestion.

3. As the temperature increases, frequency of ingestion increases from zero at 0° C to a maximum at 25°, and then decreases to zero at about 40°. There is some evidence of a median minimum at 30°.

4. As the hydrogen ion concentration decreases in solutions in which sodium is the only metallic cation, frequency of ingestion increases from zero at pH 4.4 to a maximum at approximately pH 6.0, then decreases to zero at pH 7.6 to 8.3. There is strong indication of a median minimum between pH 6.5 and 7.0. The results indicate that in solutions in which the concentration of sodium is 0.005 N, frequency of ingestion is closely correlated with the hydrogen ion concentration, but not in solutions in which the concentration of sodium is 0.002 N.

5. The relation between hydrogen ion concentration and frequency of ingestion in solutions in which potassium is the only metallic cation is similar to that in solutions in which sodium is the only metallic cation, but the range over which ingestion occurs is much shorter and the frequency of ingestion is much lower.

6. In solutions in which calcium is the only metallic cation, frequency of ingestion increases from about 0.9 chilomonads per minute at pH 4.7 to a maximum of 1.8 chilomonads per minute at approximately pH 6.5, then decreases very slowly until pH 8.0 is reached, and then drops rapidly to zero.

7. In solutions in which sodium and calcium are the only metallic cations, frequency of ingestion increases very rapidly from zero at pH 4.5, to a maximum at pH 6.2 then decreases to a marked median minimum at pH 7.0, then increases to a secondary maximum approximately at pH 7.7, then decreases nearly to zero at pH 8.4. The median minimum and the secondary maximum in the alkaline region of the range are much more pronounced in solutions containing Na 0.005 N and Ca 0.003 M than in solutions containing Na 0.005 N and Ca 0.001 M.

8. In solutions of NaCl, as the concentration increased, frequency of ingestion increased very rapidly from about 0.49 chilomonads per minute at 0.0003 M, to a maximum of 1.54 chilomonads per minute at 0.0008 M, and then decreased slowly to zero at 0.03 M.

9. The relation between concentration and frequency of ingestion in solutions of KCl is essentially the same as in solutions of NaCl; but the frequency of ingestion is much

lower, and the range over which ingestion occurs is only about one-third as long, the maximum concentration being 0.03 M in the one and only 0.01 M in the other.

10. In solutions of $MgCl_2$, as the concentration increases, the frequency of ingestion increases rapidly from 0.59 chilomonads per minute at 0.0001 M, to a maximum of 0.83 chilomonads at 0.0005 M, and then decreases gradually to zero at 0.005 M.

11. The relation between concentration and frequency of ingestion in solutions of $CaCl_2$ is essentially the same as in solutions of $MgCl_2$ except that frequency in the lowest concentration tested is considerably higher.

12. The range of concentration over which ingestion occurs in solutions of $MgCl_2$ or $CaCl_2$ is only about one-sixth as long as that over which it occurs in solutions of NaCl. The cause of this difference is not clear.

13. The relation between temperature, salts, and hydrogen ion concentration and the frequency of ingestion of food is essentially the same as the relation between these environmental factors and rate of locomotion. Frequency of ingestion and rate of locomotion are therefore closely correlated.

14. Frequency of ingestion of chilomonads by *A. proteus* and *A. dubia* is zero in solutions of lactose in all concentrations, but is it higher in Hahnert solution plus lactose than in Hahnert solution without lactose. This indicates that osmosis is involved in feeding.

15. The frequency of ingestion is much higher in Chalkley solution than in Hahnert solution plus lactose 0.003 M (osmotically equal to Chalkley solution). This is doubtless due to the fact that the former contains NaCl and the latter does not.

16. The frequency of ingestion is much higher in *A. dubia* than in *A. proteus*. The maximum number ingested by an individual of the former was 100 in 15 minutes, 195 in 30 minutes, 366 in 60 minutes, and 405 in 120 minutes; and by one of the latter, only 48 in 15 minutes, 95 in 30 minutes, 130 in 60 minutes, and 250 in 120 minutes.

LITERATURE CITED

BLASDALE, W. C. 1931. The fundamentals of quantitative analysis. New York. Pp. 403.

CLARK, W. 1928. The determination of hydrogen ions. Baltimore. Pp. 717.

FARBER, J. E., and YOUNGBURG, G. 1932. Determination of phosphates in waters. Ind. and Eng. Chem., 4:107–11.

FENN, W. O. 1921. The phagocytosis of solid particles. II. Carbon. Jour. of Gen. Physiol., 3:465–82.

HAHNERT, W. F. 1932. Studies on the chemical needs of *Amoeba proteus:* A culture method. Biol. Bull., 62:205–11.

HARGITT, G. T., and FRAY, W. W. 1917. The growth of *Paramecia* in pure cultures of bacteria. Jour. Exp. Zoöl., 22: 421–25.

HEILBRUNN, L. V., and DAUGHERTY, KATHRYN. 1932. The action of sodium, potassium, calcium and magnesium ions on the plasmagel of *Amoeba proteus*. Physiol. Zoöl., 5:254–74.

HOPKINS, D. L. 1928. The effect of certain physical and chemical factors in locomotion and other life processes in *Amoeba proteus*. Jour. Morph. and Physiol., 45: 97–119.

MAST, S. O. 1926. Structure, movement, locomotion and stimulation in *Amoeba*. *Ibid.*, 61:347–425.

———. 1929. Mechanics of locomotion in *Amoeba proteus* with special reference to the factors involved in attachment to the substratum. Protoplasma, 8:344–77.

MAST, S. O., and HAHNERT, W. F. 1935. Feeding, digestion, and starvation in *Amoeba proteus*. Physiol. Zoöl., 8:252–72.

MAST, S. O., and PROSSER, C. L. 1932. The effect of temperature, salts, hydrogen ion concentration on rupture of the plasmagel sheet, rate of locomotion, and gel/sol ration in *Amoeba proteus*. Jour. Cell. and Comp. Physiol., 1:333–54.

METALNIKOW, S. 1912. Contributions à l'étude de la digestion intracellulaire chez les protozoaries. Archiv. de zool. exp., 9:373–498.

PHILLIPS, RUTH L. 1922. The growth of *Paramecium* in infusions of known bacterial content. Jour. Exp. Zool., 36:135–83

PITTS, R. F. 1932. Constant temperature apparatus adapted for use on the microscopic stage. Science, N.S., 76:626–27.

PITTS, R. F., and MAST, S. O. 1934. The relation between inorganic salt concentration, hydrogen ion concentration, and physiological processes in *Amoeba proteus*. II. Rate of locomotion, gel/sol ratio, and hydrogen ion concentration in solutions of single salts. Jour Cell. and Comp. Physiol., 4:237–56.

———. 1934. The relation between inorganic salt concentration, hydrogen ion concentration, and physiological processes in *Amoeba proteus*. III. The interaction between salts (antagonism) in relation to hydrogen ion concentration and salt concentration. *Ibid.*, pp. 435–55

SCHAEFFER, A. A. 1910. Selection of food in *Stentor coeruleus* Ehr. Jour. Exp. Zool., 8:75–132.

———. 1916. On the feeding habits of *Amoeba*. *Ibid.*, 20:529–48.

SCHWITALLA, A. M. 1925. The rate of locomotion in *Amoeba* at different temperatures. Jour. Morph. and Physiol., 41:45–58.

THE EFFECT OF CHEMICALS AND TEMPERATURE ON REVERSAL IN CILIARY ACTION IN PARAMECIUM[1]

(One figure)

JOSEPH F. OLIPHANT
Stanford University

REVERSAL in direction of the effective stroke of the cilia is very common in the protozoa and occurs frequently in the metazoa, but only certain phases of it have been adequately investigated, and few attempts have been made to ascertain the physiological and environmental factors involved in the phenomenon.

Jennings (1899*a*) found that a variety of stimuli (chemical, mechanical, electrical, and thermal) cause *Paramecium* momentarily to reverse its direction of locomotion. He further noted (1899, p. 34) that in paramecia put into a 1 per cent solution of KI the action of the cilia reverses for 6–8 minutes.

Parker (1896, 1905*a*, 1905*b*) found the action of the cilia on the oral disk of *Metridium marginatum* reverses in response to dilute crab-meat juice, a sea-water extract of rubber, 2, 3, 4, and 5 per cent solutions of KCl in sea water, a 5/8 M solution of NaCl containing M/15 or M/30 creatin, to peptone or deutero-albumine, and asparaginic acid. He did not obtain reversal using NaCl alone, LiCl, NHCl, $NaNO_3$, a number of sugars, urea, creatinine, or chemically inert substances such as bits of filter paper or sand. Before he had worked with organic compounds other than crab-meat juice, he said (1905*a*, p. 13): "Reversal is due to potassium ions or to some organic combination containing potassium ions." After having observed the effects of various organic compounds, he modified these statements and said (1905*b*, p. 5): "The reversal.... is due cheifly to molecular action, though potassium ions if sufficiently concentrated will cause reversal."

Merton (1923) obtained reversal of ciliary action in *Stentor coeruleus* in solutions containing Na and K ions but did not obtain reversal in solutions of $CaCl_2$, $MgSO_4$, and $MgCl_2$. He suggested that the reversal is due to the Na and K ions and concluded (p. 13):

> Wenn der Zusatz von K oder Na Ionien eine Umkehr der Flimmerbewegung hervorruft, so besteht die Möglichkeit, das unter naturlichen Bedingungen diese Ionien selbst in dem Stentor wirksam sind. Es kann aber auch und das ist wahrschleinlicher ein unbekannter Stuff diese Reaktion hervorrufen und nur im Versuch durch diese Kationen ersetzt werden.

Merton (1935) found in further studies with *Stentor* that a number of salts of K, Na, Rb, Ce, and NH_4 induce reversal in the action of the cilia. Reversal, however, was not obtained with calcium salts. He says that reversal results from some specific action of the alkali metals, though he does not say what the specific action is.

Mast and Nadler (1926) investigated the relation between fifty-six different chemicals in the environment and reversal in ciliary action in *Paramecium caudatum*. By observing the direction of locomotion, they ascertained qualitatively the effect of the chemicals in inducing reversal. They found that all the univalent salts and hydrates tested induce reversal except $(NH_4)_2SO_4$ and $(NH_4)C_2H_3O_2$ but none of the bivalent and trivalent cation salts and hydrates except $CaHPO_4$, $MgHPO_4$ and $Ba(OH)_2$. Polyvalent cation salts were found to neutralize the effect of monovalent cation salts. They conclude (p. 117): "Ciliary reversal is associated with differential absorption of cations and subsequent changes in electrical potential, but there are also other factors involved."

---

[1] Contribution from the Zoölogical Laboratory, Johns Hopkins University. The author wishes to acknowledge his indebtedness to Professor S. O. Mast for valuable suggestions concerning the experiments and for valuable assistance in the preparation of the manuscript.

Eisenberg-Hamburg (1932) reported a *Ruckwärtsbewegungreaktion* when *Paramecium* is introduced into solutions of salts of Sr, Rb, and Ce. She maintains that this reaction consists of a series of backward and forward movements extending over considerable periods of time and that the reversed phase is in each instance of very short duration. The reaction was also obtained, though not in such a characteristic manner, in Ba salts but was not obtained in Mg, Ca, Cd, Na, K, and Fe salts. Similar results were obtained with *Colpidium*, *Stentor*, and *Spirostomum*. She concludes (p. 123): "Die Rückwärtsbewegungreaktion bildet somit eine Reflexbewegung des normalen Infusors, hervorgerufen durch die Anwesenheit des Sr Kations [and by inference other cations] dessen chemisch-physikalischen Wirkung aller Wahrscheinlichkeit nach die Kolloide der Zelle angreift."

It is the object of the present investigation to ascertain more precisely the relation between monovalent cation salts in the environment and reversal in the effective beat of cilia with the view of throwing light on the physiological factors involved in ciliary reversal.

MATERIALS AND METHODS

For this investigation *Paramecium multimicronucleatum* was chosen owing to its size and the readiness with which it lends itself to culture and experimentation. The specimens used were all obtained from a clone cultured as follows: 7 gr. of timothy hay in a liter of Chattolanee spring water were heated and kept at the boiling-point for 3 minutes; then 200 cc. of this infusion were poured into each of five Erlenmeyer flasks, which were then stoppered with cotton plugs and left 12 hours, after which 5 cc. of old infusion containing paramecia were added to each flask. New cultures were prepared each week, and no culture was used for experimentation until after it had stood for a week. By this time the hydrogen-ion concentration of the culture was pH 6.9 ± 0.1. The temperature of the room in which the cultures were grown varied between 21° and 23° C., inclusive. By this method animals in nearly the same physiological condition were obtained for the experiments.

The chemicals used were Kahlbaum's "pro-analyse" and Merck's blue-label Reagent. The chemical solutions were made with Chattolanee spring water. This spring water was found to be more favorable than any other solution used. Distilled water has been used by other investigators, but it is not as satisfactory as spring water, for, when *Paramecium* is transferred from a culture medium to distilled water, action of the cilia is reversed. It is therefore necessary to acclimatize specimens to distilled water before making tests of effects of chemicals. This acclimatization must be done by gradual dilution over a period of 12 hours or more and even then there are not infrequently produced ruptures of the ectosarc by the entrance of water.

Merton (1935) used tap water in his studies on *Stentor*. In preliminary experiments of the present work it was found that various tap waters and spring waters can be used but that few of them are satisfactory because of great seasonal variation in content.

Chattolanee spring water is obtained from covered springs, and chemical analyses show that its variation in total salt content throughout the year is only about 1 milligram per cent.[2] It tends to act as a natural buffer, and it has no appreciable effect on *Paramecium*. Thoroughly washed specimens live in it for as long as 10 days.

In making the tests, paramecia were first thoroughly washed in spring water, transferred to pure spring water, and left for an hour before being used for experimentation. To ascertain the effects of chemicals on ciliary reversal, 2.5 cc. of solution to be tested were put into a Columbia pyrex glass culture dish on the stage of a dissecting binocular microscope. One of the washed paramecia was taken up in a capillary pipette, care being exercised to take as little water as possible, and dropped into the test solution. The specimen was then observed continuously until reversed action of the cilia ceased and the duration of reversal measured with a stop watch. This was repeated with other specimens as often as was desired.

Numerous experiments by various investigators demonstrate that reversal in the action of the cilia in *Paramecium* is sometimes confined to localized regions of the body. In all the experiments presented here ciliary reversal was only recorded as having occurred if the specimens actually swam backward for a time measurable with a stop watch.

The method described above was slightly modified in some experiments as indicated in the following pages.

RELATION BETWEEN CONCENTRATION OF MONOVALENT CATION SALTS AND REVERSAL IN CILIARY ACTION

The time of backward swimming was ascertained for ten specimens in each of a series of concentrations of the chlorides, iodides, and bromides of sodium, potassium, lithium, and ammonium. The hydrogen-ion concentration of the chemical solutions used was pH 7.4 ± 0.1, that of the culture medium from which the organisms were taken pH 6.9 ± 0.1, and that of the wash solution to which the specimens were adapted pH 7.4. The temperature varied between 21° and 23°C.

In molar solutions in NH_4Br, NH_4Cl, and NH_4I the specimens died immediately without reversal, while in all the other salts they died in 1–2 seconds and swam backward continuously. In M/5 and M/10 NH_4I death was immediate, and there was no reversal. In M/5 and M/10 LiI and LiBr death occurred in about 10 seconds, and the paramecia swam backward continuously. In M/5 concentrations of the other salts death occurred in 2–8 minutes, and there was an initial period of reversed ciliary action which was followed by momentary recovery and normal forward movement after which there were repeated reversals and recoveries until death. In M/5 NaBr, for example, the animals swam backward 9.45 ± seconds then forward 3–5 seconds then backward 0.5–1.0 seconds then forward 1–3 seconds and then continued to reverse at this frequency about 2–3.5 minutes, i.e., until they died, which usually, but not always, occurred in a reversed phase of about 30 seconds' duration. This type of behavior occurred in the other sodium salts and in M/5 and M/10 LiCl. In higher concentrations the animals, immediately before they died, revolved in a plane about the posterior end without rotating on the longitudinal axis. In other concentrations of all salts tested the initial resumption of forward movement was seldom followed by subsequent reversals. Recovery from a reversed condition in these concentrations was always preceded by a type of behavior in which the anterior end of the animal moved in a great circle while the posterior end moved in a small circle. Forward movement in recovered specimens was a little slower than in animals not subjected to the action of a chemical.

---

[2] Composition of Chattolanee spring water in grams of salt per liter: NaCl, 0.0034; Na_2CO_3; 0.00013; K_2SO_4, 0.0017; $CaSO_4$, 0.00007; $MgCO_3$, 0.020; $CaCO_3$, 0.052; silica, 0.0092; oxides of Fe and Al, 0.00066.

In M/400 of all the salts tested except those of ammonium there was a pronounced motor response accompanied by momentary reversal of such short duration that it could not be measured with a stop watch. In M/1,000 of the various salts except those of ammonium paramecia gave a slight motor response, but in no instance was there anything that could be considered true ciliary reversal. Similar results were obtained in ammonium salts M/50–M/150.

The results of one complete series of experiments are summarized in Table 1 and Figure 1. The table and figure show that in solutions of chlorides, iodides, and bromides of sodium, potassium, lithium, and ammonium duration of reversed ciliary action varies directly with concentration of the salt and that the order of effectiveness of the monovalent cations in inducing ciliary reversal is K > Li > Na > NH_4. A typical series of readings from which the average duration of reversal was computed, the one obtained in M/10 NaCl, the first salt in the table, follows: 11.1, 10.8, 11.2, 12.5, 11.9, 12.0, 10.5, 10.1, 8.4, and 9.6 seconds. The variation in individual readings was somewhat greater in bromides and iodides of potassium and lithium. This is shown typically by the results obtained with M/10 KBr which are 28.2, 32.5, 28.8, 27.6, 27.9, 27.1, 44.3, 25.6, 37.5, and 36.1 seconds. These series indicate considerable variation; however, in columns 4, 5, and 6 of the table it is revealed that the standard deviation is small in comparison with the average time of reversed ciliary action and that the probable error is very small, rarely exceeding 6 per cent. The standard deviation and percentage of probable error are greatest in the iodides, less in the bromides, and least in the chlorides. The reason for this variation is not clear.

All the experiments summarized in Table 1 and Figure 1 were repeated twice, and the results obtained are qualitatively and quantitatively in agreement with those presented.

The conclusions of these experiments are in accord with the conclusion of Eisenberg-Hamburg (1932) that the *Rückwärtsbewegungreaktion* is greater, the greater the concentration of the salt inducing it, and they are supported by the results obtained by Merton (1935) on *Stentor*, which indicate that duration of reversal varies directly with concentration; but they are not in accord with the results obtained by Mast and Nadler on observations on *P. caudatum*. They say (1926, p. 116):

> The duration of reversed action varies with the kind of salt and with the concentration. In KCl as the concentration decreases, the duration of this action increases from zero at M/10 to a maximum of about 40 seconds at M/750, then decreases to zero at M/2000. In NaCl it increases from zero at M to a maximum of about 14 seconds at M/500, then decreases to zero at M/2200. In NH_4Cl it increases from zero at M/50 to a maximum of about 10 seconds at M/1000, then decreases to zero at M/2200. In LiCl it increases very rapidly from zero at M/5 to a maximum of about 55 seconds at M/10, then decreases slowly to zero at M/2000.

The results presented above are obviously not in accord with these claims, for they show that in solutions of KCl, LiCl, and NH_4Cl duration of reversed ciliary action varied directly with concentration and that no reversal in ciliary action was obtained in concentrations between M/500 and M/750, the optimum for reversal found by Mast and Nadler.

TABLE 1

RELATION BETWEEN DURATION OF REVERSED CILIARY ACTION IN *Paramecium* AND CONCENTRATION OF MONOVALENT CATION SALT INDUCING REVERSAL

"Average," time of reversal for ten specimens; S.D., standard deviation; P.E., probable error; percentage P.E., percentage probable error of the average duration of reversal.

Salt	Concentration	Average	S.D.	P.E.	Percentage P.E.
NaCl	M/10	10.81	1.81	0.25	2.3
	M/20	7.49	0.80	0.16	2.1
	M/50	5.73	0.87	0.18	3.1
	M/100	6.04	1.40	0.29	4.7
	M/200	4.26	0.77	0.16	3.7
KCl	M/10	43.82	3.03	0.64	1.4
	M/20	30.05	3.59	0.74	2.4
	M/50	14.22	3.56	0.74	5.2
	M/100	3.92	0.95	0.20	5.1
	M/200	1.00	Not calculated		....
LiCl	M/10	13.22	1.88	0.40	3.1
	M/20	9.00	1.24	0.26	2.8
	M/50	7.52	1.80	0.38	5.0
	M/100	5.28	0.86	0.18	3.4
	M/200	3.52	0.55	0.11	3.1
NH_4Cl	M/5	21.20	2.25	0.47	2.2
	M/10	8.70	1.78	0.37	4.2
	M/20	2.10	0.38	0.80	3.8
	M/50	1.00	Not calculated		....
	M/100	?	....	....	....
NaBr	M/10	8.12	1.73	0.33	4.0
	M/20	5.63	1.24	0.21	3.7
	M/50	3.74	1.11	0.25	6.6
	M/100	1.00	Not calculated		....
	M/200	?	....	....	....
KBr	M/10	31.56	5.92	1.26	5.1
	M/20	29.78	8.62	1.80	6.0
	M/50	9.25	2.27	0.48	5.1
	M/100	3.14	0.78	0.16	3.9
	M/200	1.00	Not calculated		....
LiBr	M/10	8.59	1.53	0.26	3.0
	M/20....	13.25	5.15	1.63	12.1
	M/50	5.71	0.20	0.13	2.1
	M/100	3.89	1.05	0.22	5.6
	M/200	1.00	Not calculated		....
NH_4Br	M/5	10.62	2.81	0.59	5.5
	M/10	3.97	1.65	0.34	8.3
	M/20	1.00	Not calculated		....
	M/50	?	....	....	....
NaI	M/10	7.53	2.78	0.59	7.9
	M/20	4.74	0.70	0.14	2.9
	M/50	3.47	1.20	0.24	6.8
	M/100	2.95	1.02	0.21	7.1
	M/200	1.00	Not calculated		....

TABLE 1—*Continued*

Salt	Concentration	Average	S.D.	P.E.	Percentage P.E.
KI..	M/10	53.36	14.37	3.06	5.7
	M/20	49.92	15.26	3 24	6.4
	M/50	12.57	3.46	0.73	5.7
	M/100	4 16	1.02	0 21	5 0
	M/200	1.00	Not calculated		....
LiI	M/10	14.09	3.95	0.84	5.2
	M/20	13 95	4 25	0 90	6.4
	M/50	6.02	1 95	0.41	6.8
	M/100	4.43	1.70	0.36	8.1
	M/200	1.00	Not calculated		....
NH_4I.............	M/10	Death without reversal			....
	M/20	5 35	1.35	0.24	4.4
	M/50	8.22	1.03	0.21	3.2
	M/100	6.49	0.97	0.20	3.0
	M/200	?	....	....	....

The differences in results of the two investigations may have been due to differences in physiological states of the organisms traceable to conditions in the infusions in which they were grown and to adaptation to different wash solutions. Mast and Nadler used *P. caudatum* adapted to distilled water. The hydrogen-ion concentrations were not controlled, and the temperatures are not given. The differences are not due to the fact that different species were used, for in tests of *P. caudatum* in KI, KCl, and KBr under conditions of the present investigation results were obtained which are essentially the same as those obtained with *P. multimicronucleatum.*

THE ROLE OF ANIONS IN CILIARY REVERSAL

The possible role of the anions used in the present experiments was considered. When the data presented in Table 1 are compared, it is found that the difference in effect produced by like concentrations of chloride, bromide, and iodide having the same cation is in all instances slight, and it is impossible to recognize a consistent order in which these anions affect ciliary reversal.

To investigate further the possible role of anions, the effect of some bivalent cation salts was tested. Calcium and magnesium were used because they are closely related chemically to the alkali metals, whose salts induce reversal, and they are among the more abundant bivalent cations found in and having pronounced effects upon various types of protoplasm. Chlorides, bromides, and iodides of calcium and magnesium were tested from M/1 to M/20,000. In concentrations from those not immediately lethal about M/250 up to M/2,500 of each salt various types of motor reactions were observed, but in no instance was there anything that could be considered true ciliary reversal. These experiments indicate that calcium and magnesium ions do not induce reversal in ciliary action in *Paramecium* and that chlorine, bromine, and iodine ions do not induce reversal and that their action in reversal is quantitative. No other anions were investigated. However, Merton (1935) in experiments on *Stentor* presents evidence which indicates that some salts of potassium are more effective in inducing ciliary reversal than others. He lists the anions used above in the order Cl > I > Br.

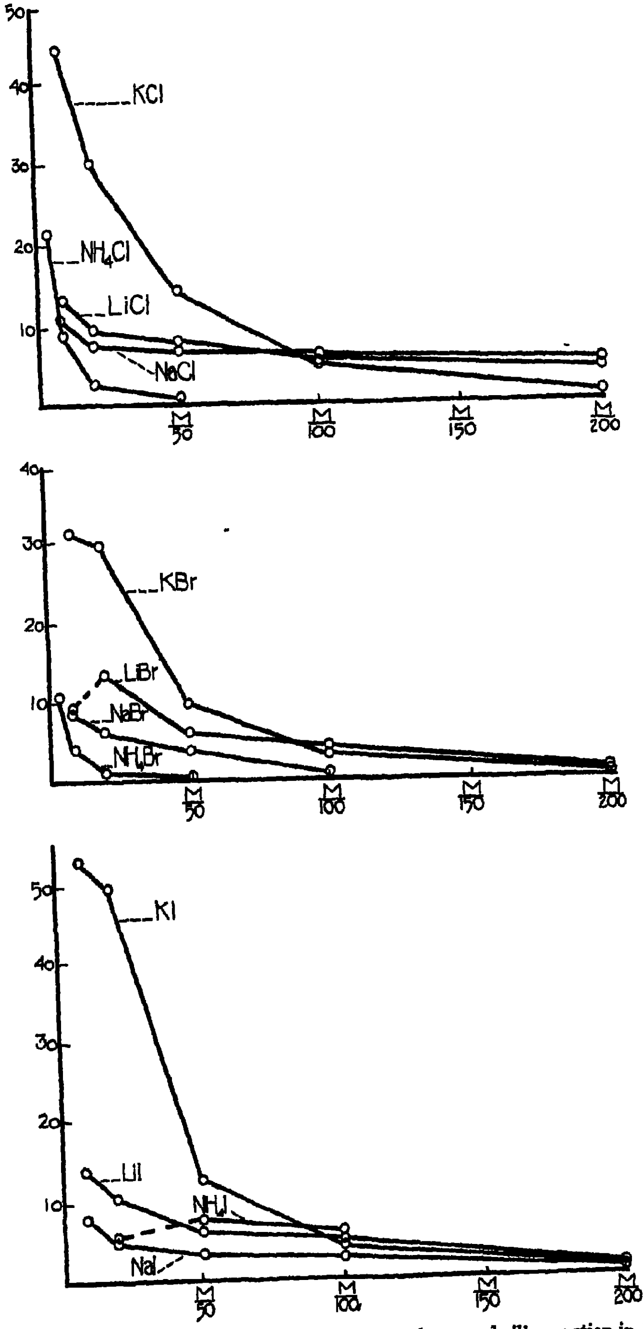


Fig. 1.—Graphs showing the relation between duration of reversed ciliary action in *Paramecium* and concentration of various monovalent cation salts inducing reversal. Ordinates, time of reversed ciliary action in seconds; abcissae, concentration of salt.

THE EFFECT OF ADAPTATION ON CILIARY REVERSAL INDUCED BY SOLUTIONS OF MONOVALENT CATION SALTS

The question whether ciliary reversal in *Paramecium* is dependent on the absolute concentration of monovalent cation salt in the environment or upon rate of change (increase) in concentration has a direct bearing on adaptation. It was investigated as follows: Numerous paramecia were thoroughly washed in spring water; then 6 cc. of solution containing washed paramecia were added to 6 cc. of a monovalent cation salt solution M/20 in a 25 cc. stender dish. The dish was then covered and left for 18 hours. These paramecia and others which were washed but had not been in the salt solution were then tested for reversal in ciliary action in a number of concentrations of the salt to which the experimental animals had been·exposed. This experiment was repeated with the chlorides, iodides, and bromides of sodium, lithium, and potassium. Care was exercised to adapt approximately the same number of organisms in each experiment.

It was found that *Paramecium* becomes adapted (no longer swims backward) to M/40 solutions of all salts tested in less than 18 hours, otherwise the adapted paramecia behaved essentially like those which were not adapted; however, the behavior was not the same in all salts. The paramecia adapted to lithium and potassium salts moved slightly slower and were darker and much more vacuolated than the controls. A microscopic examination revealed an apparent increase in protoplasmic consistency, and there was evidence of slight plasmolysis. Specimens adapted to sodium salts behaved more nearly like the controls; however, they were darker in appearance and more vacuolated. No difference could be detected between paramecia adapted to chlorides, iodides, and bromides having the same cation. After paramecia had remained two days and longer in lithium and potassium salts, movement was accompanied by active changes in shape, was very slow in comparison with controls, and reversals in the direction of rotation on the longitudinal axis increased. The paramecia in lithium salts died first, then those in potassium salts, then those in sodium salts. The order in which the cations produce death therefore seems to be in no way correlated with the order in which they induce reversal in ciliary action. Observations were not made at sufficiently frequent intervals to ascertain the order of the anions.

The rate of reversed beat of the cilia was observed to differ in the different salts, but no actual measurements were made. However, it could be seen that it was greatest in lithium, less in potassium, and least in sodium salts. This is not in accord with the order of effectiveness of these cations in inducing ciliary reversal, but it is in accord with the order in which they produce death.

Some of the results of the experiments are presented in Table 2. This table shows that in paramecia adapted to a M/40 solution of a given monovalent cation salt ciliary reversal does not occur in lower concentrations (M/50 and M/100) but does occur in higher concentrations (M/20, M/10, and M/5). It shows further that in paramecia not adapted to a given monovalent cation salt ciliary reversal occurs in all concentrations from M/100 to M/5 and that the duration of reversed ciliary action in a given salt concentration is greater in nonadapted than in adapted specimens. These facts show that it is the rate of change (increase) and not absolute concentration of monovalent cation salt in the environment that induces reversal in the action of the cilia.

THE EFFECT OF TEMPERATURE ON CILIARY REVERSAL

Strasburger (1878) found that swarm spores which are photopositive in a given light intensity at 16°–18° C. become negative when the temperature is decreased to 4° C.

and more strongly positive when it is increased to 35° C. Massart (1891) obtained similar results in observations on *Chromulina* and Mast (1911) in observations on *Euglena*, *Chlamydomonas*, *Trachelomonas*, *Chlorogonium*, and *Volvox;* but no observations have been made on the effect of change in temperature on reversal in ciliary action.

In making this investigation, the temperature was controlled within 0.5° C. by means of temperature chambers on the stage of a low-power binocular microscope (Pitts, 1932). In making the tests, paramecia were thoroughly washed and then left 1½ hours in spring water at a given temperature in a temperature chamber on the stage of a binocular microscope. One individual was then taken up in a capillary pipette and put into 5 cc. of solution to be tested (in another temperature chamber on the stage of another microscope) at any temperature desired, then the behavior of the specimen was observed continuously until the end of the experiment. If there was ciliary reversal, the duration was measured with a stop watch. This procedure was repeated as often as desired. Observations were made on ten specimens at each temperature tested.

TABLE 2

EFFECT OF ADAPTATION TO SALT ON DURATION OF REVERSED CILIARY ACTION IN *Paramecium*.

Each number in the table is the average of the duration in seconds of reversal for ten specimens *Ins.* indicates reversal too short in duration to be measured with a stop watch.

CONCENTRATION	AVERAGE DURATION IN SECONDS OF REVERSED CILIARY ACTION								
	NaI	NaBr	NaCl	LiI	LiBr	LiCl	KI	KBr	KCl
	Paramecia adapted to M/40 Salt Solutions								
M/100	0.00	0.00	0.00	0.00	0.00	0 00	0 00	0.00	0 00
M/50	0.00	0.00	0.00	0 00	0 00	0.00	0 00	0.00	0.00
M/20	1.24	0.75	1.29	2.10	4 62	4.02	24 15	5.49	1.39
M/10	2.47	2.67	2.51	5.07	8.33	7.00	41.62	13 91	6.60
M/5	6.41	3.82	7.29	5.10	23.79	17 67	55 17	16.01	9.77
	Paramecia Not Adapted								
M/100	Ins.	Ins.	2.07	2.59	3.33	4.13	3.21	2.19	2.11
M/50	2.22	2.87	4.62	5.46	6.18	6.33	26.14	8.16	8.56
M/20	5.10	4.15	6 18	12.17	11.23	8.45	43 52	28.14	24.34
M/10	8.27	9.11	8.76	15.24	13.82	13.27	51.14	37.64	32.67
M/5	11.31	11.27	11.39	12.00	18.10	7.53	73.46	45.58	41.21

Paramecia were left in spring water at 23° C. for 1½ hours, then transferred to spring water at 7°, 15°, 23°, 30°, 40°, and 50° C. The following was observed: at 7°–30° there were violent motor reactions but no continuous backward swimming. At 40° C. there was ciliary reversal which continued 1.5 seconds. At 50° the paramecia died immediately. In paramecia adapted to 15° and 30°, respectively, similar results were obtained. The paramecia did not become adapted to 7°, 35°, and 40° C.

These experiments indicate that reversal in ciliary action occurs only after transfer to temperatures almost immediately lethal and then continues only a very short time.

Paramecia were adapted for 1½ hours to spring water at 23° C. then put into 5 cc. of various chemical solutions at 7°, 15°, 23°, 30°, 40°, and 50° C. The results obtained are presented in Table 3.

Table 3 shows that duration of reversed ciliary action in M/20 and M/50 solutions of all the salts tested varies inversely with temperature and that in a given concentration of chloride, bromide, or iodide of potassium, sodium, and lithium at a given temperature the order of effectiveness of the monovalent cations in inducing reversal is K > Li > Na.

TABLE 3

RELATION BETWEEN DURATION OF REVERSED CILIARY ACTION IN *Paramecium* IN M/20 AND M/50 SOLUTIONS OF SALTS

Each number in the table represents the average duration of reversal in seconds in ten specimens. Death was immediate without reversal.

SALT	CONCENTRATION	TEMPERATURE					
		7° C.	15° C.	23° C.	30° C.	40° C.	50° C.
KCl	M/20	68 63	42 09	28.20	20 36	16 81	Death
KCl	M/50	48.12	20 86	11 58	6 25	3 80	"
LiCl	M/20	77.31	32 80	8 94	4 89	3 71	"
LiCl	M/50	65 91	24 14	6.57	3 69	2 23	"
NaCl	M/20	23 29	11.79	5 60	2.80	1 61	"
NaCl	M/50	18 94	9 53	4.22	1.86	1.24	"
KBr	M/20	83.89	52 44	27 39	19 53	8 29	"
KBr	M/50	39.27	21 63	12 15	4.81	3.20	"
LiBr	M/20	78.33	29 56	11 72	5.32	4.18	"
LiBr	M/50	51.12	19.27	6 52	5 15	4.78	"
NaBr	M/20	22.89	11 91	5 37	3.99	1.76	"
NaBr	M/50	17.62	9 21	4 47	2.26	2.15	"
KI	M/20	97.30	44.21	30 76	12.92	8 22	"
KI	M/50	43 97	16.81	11.21	5.38	3.36	"
LiI	M/20	87.49	31.20	13.18	5.26	3.67	"
LiI	M/50	44.34	16 51	7 16	2.94	2.72	"
NaI	M/20	35.62	15 41	7.06	4.39	2.69	"
NaI	M/50	26.43	9.27	4.21	3 53	2.47	"

Comparison of relation between duration of reversal in a given concentration of chlorides, bromides, and iodides having the same cation and temperature indicates that, if these anions affect ciliary reversal, there is no constant or significant order in which they affect it. The similarity of effects produced by salts of the same cation but different anions is particularly striking in the salts of lithium given in Table 3.

There were marked differences in rate at which paramecia swam backward at different temperatures. The rate was very slow at 7° C., somewhat more rapid at 15° C., rapid at 23°, very rapid at 30°, and extremely rapid at 40° C. Actual measurements were not made, but careful observation indicates that it is probably directly proportional to the temperature.

Paramecia exposed to the lower temperatures became darker in appearance and more vacuolated, and the granular nature of the endoplasm was more pronounced. Death in high temperatures was accompanied by increased vacuolation change in shape and coagulation of the cytoplasm.

Similar results were obtained with paramecia adapted to 15° and 30° C.

DISCUSSION

It is demonstrated above that *Paramecium* reacts to increase in monovalent cation salt concentration in the environment by reversal in action of the cilia but does not react to increase in bivalent cation salt concentration. This difference in response must be due to some difference in the effect of monovalent and bivalent cations on the protoplasm.

Heilbrunn (1923) found chlorides of calcium and magnesium cause a decrease, while chlorides of potassium, sodium, and ammonium cause an increase in protoplasmic viscosity of eggs of *Arbacia*. He (1923) obtained similar results with *Stentor*, as did Heilbrunn and Daugherty (1931) in *Amoeba dubia*. Cholodnyj (1923) found potassium and lithium produce marked increase in protoplasmic viscosity in root hairs of *Trianea* but that calcium and magnesium have no appreciable effect. Weber (1924) reached similar conclusions in reference to effects of sodium and calcium chloride on *Spirogyra*, and Spek (1921) maintains that viscosity of the protoplasm in *Actinosphaerium* is increased by cations in the following order: Li > K > Na > Ca > Mg. Spek (1920) found LiBr, LiCl, and KOH cause *Paramecium* to increase in volume, and he attributes this to a swelling action of the colloid resulting in increased viscosity. There is consequently, considerable evidence which indicates that monovalent cations induce increase and bivalent cations decrease in viscosity of protoplasm.

If this is true and the cilia are under control of the neuromotor apparatus, it is conceivable that an increase in viscosity of the cytoplasm could produce impulses in the neuromotor apparatus resulting in reversal in the action of the cilia.

That reversal is associated with increased viscosity is further suggested by the observation that reversal in response to temperature occurs only at temperatures almost immediately lethal, it being well established that there is a marked increase in viscosity preceding death at high temperatures. It is also shown that duration of reversal induced by chemicals varies inversely with temperature, and there are many observations which indicate that viscosity of protoplasm varies inversely with temperature

Precisely how environmental changes which have been observed to induce reversal in the action of cilia effect reversal and the physiological factors involved cannot be completely explained on a basis of our present knowledge. Factors such as changes in the electric potential at the surface, or in permeability and consistency of the surface layer of the organism, or chemical changes in the receptors or elsewhere in the organism may be involved as has been suggested. It is believed, however, that there is considerable evidence to indicate that reversal is associated with increase in viscosity of the cytoplasm. A more comprehensive study of this problem is being undertaken.

SUMMARY

1. The chlorides, bromides, and iodides of potassium, lithium, sodium, and ammonium induce reversal in the direction of the effective beat of the cilia of *Paramecium*. The chlorides, bromides, and iodides of calcium and magnesium do not.

2. The duration of reversed ciliary action varies directly with concentration of the salt which induces it.

3. The effect of monovalent salts on reversal is primarily due to the action of the cations. If the anions have any effect, it is quantitative and is secondary to that of the cations.

4. The order of effectiveness of the monovalent cations in inducing reversal is K > Li > Na > NH_4.

5. Paramecia which have been 18 hours in a given concentration of chloride, bromide, and iodide of potassium, lithium, and sodium become adapted and do not exhibit ciliary reversal when transferred to solutions of lesser concentration but do reverse to solutions of higher concentration. Duration of reversal in adapted is less than in nonadapted specimens. Ciliary reversal is dependent upon rate of increase in concentration of monovalent cation salt in the environment.

6. The order in which the monovalent cations produce death in *Paramecium* is Li > K > Na.

7. Prolonged exposure to M/40 lithium and potassium salts frequently results in change in direction of rotation on the longitudinal axis in forward locomotion and active changes in shape during locomotion.

8. Change in temperature which is not lethal does not induce reversal in direction of the effective beat in the cilia in *Paramecium.*

9. Lethal temperatures induce reversal in the action of the cilia if death is not immediate, but the duration of the reversed action is very short.

10. The duration of reversed ciliary action in solutions of monovalent cation salts varies inversely with temperature.

11. The order of effectiveness of monovalent cations in inducing reversal is independent of temperature.

12. The rate at which the cilia of paramecia beat in reverse varies directly with temperature.

LITERATURE CITED

Cholodnyj, N. 1923. Zur Frage über die Beeinflussung des Protoplasmas durch mono und bivalente Metallionen. Beihefte Bot. Centralbl., 39: 231.

Eisenberg-Hamburg, E. 1932. Einfluss der Sr-Salze auf die Bewegung von *Paramecium caudatum.* Arch. f. Protist'kunde, 77: 108.

Heilbrunn, L. V. 1923. The colloid chemistry of protoplasm, I: General considerations; II: The electrical charges of protoplasm. Amer. Jour. Physiol., 64: 481.

Heilbrunn, L. V., and Daugherty, K. 1931. The action of sodium, potassium, calcium, and magnesium on the protoplasm of *Amoeba dubia.* Physiol. Zoöl., 4: 635.

Jennings, H. S. 1899*a*. Studies on reactions to stimuli in unicellular organisms, II: The mechanism of the motor reactions of *Paramecium.* Amer. Jour. Physiol., 2: 311.

———. 1899*b*. Studies on reactions to stimuli in unicellular organisms, IV: Laws of chemotaxis in *Paramecium. Ibid.*, 355.

Massart, J. 1891. Recherches sur les organismes inférieurs. La Sensibilité à la concentration chez les êtres unicellulaires marins. Bull. Belg. Acad., Ser. 3. 22: 148.

Mast, S. O. 1911. Light and the behavior of organisms. New York. Pp. 410.

Mast, S. O., and Nadler, J. E. 1926. Reversal in ciliary action in *Paramecium caudatum.* Jour. Morph. and Physiol., 43: 105.

Merton, H. 1923. Studien über Flimmerbewegung. Arch. f. d. ges. Physiol. 198: 1.

———. 1935. Zwangsreaktionen bei *Stentor* als Folge bestimmter Salzwirkungen. Biol. Zentrabl., 55: 268.

Parker, G. H. 1896. The reactions of *Metridium* to food and other substances. Bull. Mus. Comp. Zoöl., 29: 107.

———. 1905*a*. The reversal of ciliary movement in metazoans. Amer. Jour. Physiol., 13: 1.

———. 1905*b*. The reversal of the effective stroke of the labial cilia of sea anemones by organic substances. *Ibid.*, 14: 1.

Pitts, R. F. 1932. The relation between hydrogen ion concentration, inorganic salt concentration, and rate of locomotion in *Amoeba proteus.* Johns Hopkins University dissertation. Pp. 191.

Spek, J. 1920. Experimentelle Beiträge zur Kolloid-Chemie der Zellteilung. Kolloidchem. Beihefte, 12: 1.

———. 1921. Der Einfluss der Salze auf die Plasmokolloide von *Actinosphaerium Eichorni.* Acta. Zool. (Stockholm), 2: 153.

Strasburger, E. 1878. Wirkung des Lichtes und der Wärme auf Schwärmsporen. Jena Zeitsch., N.F., 12: 551.

Weber, F. 1924. Plasmolyseform und Protoplasmaviskosität. Österreich Bot. Zeitsch., 73: 261.

THE INFLUENCE OF CERTAIN PLANT HORMONES ON GROWTH OF PROTOZOA[1]

(Six figures)

ALFRED M. ELLIOTT
Biological Laboratories, Stanford University, and State Teachers
College, Bemidji, Minnesota

IT IS generally agreed that growth of stems and leaves of higher plants is stimulated by plant hormones, while growth of roots, saprophytic plants (e.g., bacteria, yeasts, and molds), and animal cells is either unaffected or else retarded in some cases. Whether this difference in effect is dependent upon some definite correlation between the presence of chlorophyll and the activity of phytohormones is not yet known, although the available evidence favors such an assumption.

The possibility that there might be some intermediate point between typical plant and animal cells at which the phytohormones lose their stimulating effect suggested a comparison of the influence of several synthetic phytohormones on the growth of three protozoa whose nutritional requirements are fairly well known. The three species selected—the chlorophyll-bearing *Euglena gracilis; Khawkinea halli*, without chlorophyll but otherwise similar to *Euglena* to which it is related; and the ciliate, *Colpidium striatum*—represent adaptations to three different methods of nutrition: holophytic. saprozoic, and holozoic, respectively. If the accelerating effects of phytohormones are dependent upon presence of chlorophyll, there should be no such effect in the case of *C. striatum* or even with *K. halli.*

MATERIAL AND METHODS

The three species of protozoa used in this investigation were maintained in bacteria-free cultures, purity being determined by periodic bacteriological tests. The strains of *E. gracilis* and *K. halli* were obtained from Professor R. P. Hall, the latter having been isolated originally by Dr. T. L. Jahn and designated previously as *Astasia* sp. (Jahn and McKibben, 1937). The strain of *C. striatum* was isolated several years ago by the writer (Elliott, 1933).

The following plant hormones were obtained from the Eastman Kodak Company: γ-3-n-indolebutyric acid ($C_{12}H_{13}O_2N$), β-3-indolepropionic acid ($C_{11}H_{11}O_2N$), and 3-indoleacetic acid ($C_{10}H_9O_2N$), or heteroauxin. Solutions of these substances were prepared a few hours before use in order to insure maximal activity.

As a basic organic medium for *C. striatum*, Difco "tryptone" was made up in 2.0 per cent solution, a concentration near the optimum for this ciliate. For the flagellates,

---

[1] This investigation was supported in part by grants from the Minnesota Academy of Science and the American Association for the Advancement of Science.

The writer wishes to express his gratitude to Professor C. V. Taylor, in whose laboratory the preliminary experiments were performed; to Dr. P. W. Zimmerman, who suggested the three indole compounds employed in the study; to Professor R. P. Hall, who carefully read the manuscript; and to Mr. Anthony Rozycki for technical assistance.

Euglena and *Khawkinea,* the same peptone was used in 0.5 per cent concentration in the following solution:

NaCl .	0 003 gm.	$MgSO_4$	0 005 gm.
KNO_3	0 002	$FeSO_4$	Trace
$CaSO_4$	0 020	Glass distilled water	1 0 liter

In determining the effects of phytohormones in relation to pH, suitable quantities of each basic medium were adjusted to the desired pH, tubed in 8.0-cc. amounts, and then sterilized in the autoclave at 15 pounds pressure for 20 minutes. The indole solutions were made up in glass distilled water and sterilized in the autoclave for 30 minutes at 115° C. To each experimental tube 1.0 cc. of sterile indole solution was added aseptically, while the controls each received 1.0 cc. of sterile distilled water. Both experimental and control tubes were inoculated with 1.0-cc. suspensions of the organisms to be tested. The flasks from which the inoculations were made were prepared from an actively growing tube of organisms, previously cultured for several weeks and through several transfers in tryptone medium. The organisms were washed and centrifuged before addition to the flask in order to remove most of the fluid in which they were growing.

Initial pH determinations were made with representative control and experimental tubes after inoculation. The initial concentration of organisms in each series was determined by counts on samples from the stock flask, the count per cubic centimeter in the inoculated tubes then being computed. In every series the cultures were incubated at a constant temperature of 26° C. The preliminary series were maintained in a constant temperature room at Stanford University (accuracy, ± 0.1° C.) while the confirmatory series were run in a constant temperature bath at State Teachers College (accuracy, ± 0.02° C.). In the case of *Euglena* the cultures were placed at 14 inches from a continuous source of artificial illumination (100-watt electric lamp). After incubation the final pH of two or more tubes from each set was determined and the remainder were fixed for counting by the addition of 0.5 cc. of a saturated solution of trinitrophenol in 1N HCl. As described previously (Elliott, 1933), all counts were made with a Sedgwick-Rafter counting chamber and a Whipple ocular micrometer. A LaMotte colorimeter was used for pH determinations. In experiments testing the effect of different concentrations of indole compounds on the growth of *Euglena,* it was necessary to culture the flagellates in 250-cc. Erlenmeyer flasks instead of tubes. Two cubic centimeter samples were withdrawn from the flasks at regular intervals and counted.

EFFECTS OF PHYTOHORMONES IN RELATION TO pH

Since it has already been noted (Elliott, 1935*a*) that pantothenic acid accelerates growth of *C. striatum* below pH 7.0 and yet is actually toxic above the neutral point, it seemed necessary to determine whether or not the effects of phytohormones might also depend upon the pH of the medium. Consequently, tests were made over the pH range, 3.0–9.0.

In the preliminary experiments it was necessary to start with an arbitrarily selected concentration of plant hormones. The concentration first used, 1:10,000 in Series I*a* and II*a*, proved toxic and subsequent tests showed that a dilution of 1:200,000 or higher was satisfactory for stimulation of *Euglena*. Hence, dilutions of 1:200,000 were used in Series I*b*, II*b*, and III.

Series Ia (*C. striatum*).—Each indole compound was added to the basic medium in concentration of 1:10,000. The pH of different sets of experimental and control tubes was adjusted as follows: 3.5, 4.5, 5.5, 6.8, 7.6, and 8.8. The initial count was 125 ciliates per cubic centimeter. After incubation for 48 hours the pH was unchanged except for a rise of 0.2 in the tubes with an initial pH of 5.5 and 6.8. Such shifts were too small to influence the results significantly.

The final counts (Fig. 1) showed that no acceleration was produced by the phytohormones. Indoleacetic acid inhibited growth almost completely at all pH levels and no attempt was made to plot the results. Indolepropionic acid was slightly toxic at pH 5.5, while indolebutyric acid produced a decided decrease in growth at the same point. At pH 6.8 and 7.6 the former showed no significant influence while the latter showed a slightly depressed effect. The results show clearly that the inhibitory effect of these two indole compounds is much greater below pH 7.0 than above the neutral point, a correlation comparable to that reported previously (Elliott, 1935*b*) for sodium acetate and sodium butyrate.

Series Ib (*C. striatum*).—In this series the growth substances were used at a concentration of 1:200,000 in tryptone solutions at pH 3.8, 4.7, 5.7, 6.8, 7.5, and 9.0. The initial count was 200 ciliates per cubic centimeter. After incubation for 45 hours pH changes were comparable to those in Series I*a*.

The final counts (Fig. 2) showed that indolepropionic and indolebutyric acid had produced practically the same effects as in Series I*a*, while indoleacetic acid was somewhat more toxic than indolepropionic acid. Here again the effects were less pronounced above pH 7.0.

Later experiments with higher dilutions (1:10,000,000) showed results similar to those of Series I*a* and I*b*, except that the inhibitory effect of the growth substances was much less striking, as might be expected. It is clear that, within the limits of these experiments, growth of *C. striatum* is not accelerated by heteroauxin, indolepropionic acid, and indolebutyric acid. It would seem, therefore, that these growth substances, which bring about such striking responses in higher plants, are not essential in the normal growth of this ciliate protozoan.

The bimaximal growth curve so typical of *C. striatum* in tryptone medium (Elliott, 1933) was absent in both Series I*a* and I*b*. Preliminary observations suggest that this may be due to the omission of the phosphate buffer from the basic medium used in the present investigation.

Series IIa (*K. halli*).—The indole compounds were used in concentrations of 1:10,000 in the 0.5 per cent tryptone medium at pH 3.2, 4.5, 5.5, 6.5, 7.5, and 9.0. The initial count was 400 flagellates per cc. No appreciable changes in pH had occurred after a 7-day incubation period. The final counts (Fig. 3) showed that the indole compounds failed to accelerate growth at any pH, and that indoleacetic acid was highly toxic in the concentration used.

Series IIb (*K. halli*).—In this series the concentration of growth substances was reduced to 1:200,000, and the initial pH of the different sets of tubes was as follows: 3.2, 4.5, 5.5, 6.7, 7.4, and 8.8. The initial count was 630. Cultures were incubated for 7 days and then fixed for counting. Final counts (Fig. 4) again failed to show an acceleration of growth, although the inhibitory effects were less marked than in Series II*a*. Comparable experiments with the hormones in dilutions of 1:10,000,000 likewise showed no acceleration of growth. It seems certain, therefore, that the indole compounds tested do not accelerate growth of *K. halli*.

Series III (*E. gracilis*).—In view of the previous experience with *Colpidium* and *Khawkinea* the phytohormones were used at a concentration of 1:200,000 in 0.5 per

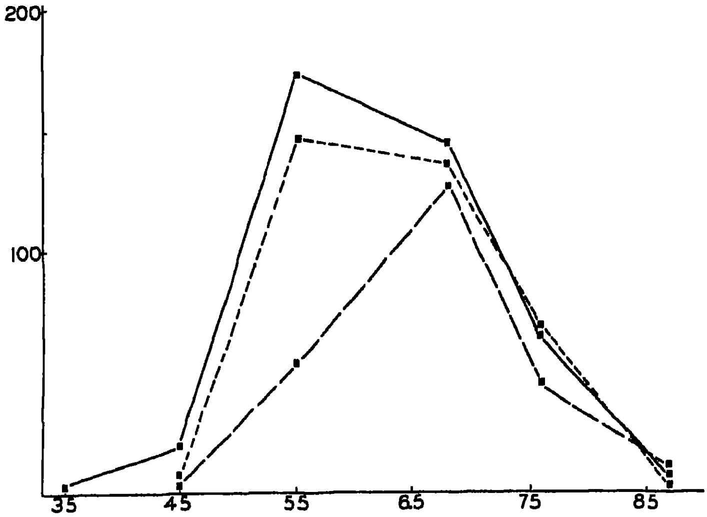


Fig. 1.—Series I*a*. Concentration of ciliates in thousands per cubic centimeter are plotted against initial pH. Cultures are indicated as follows: Control (——), indolepropionic acid (- - - - -) and indolebutyric acid (— —). Concentration of indole compounds is 1:10,000.

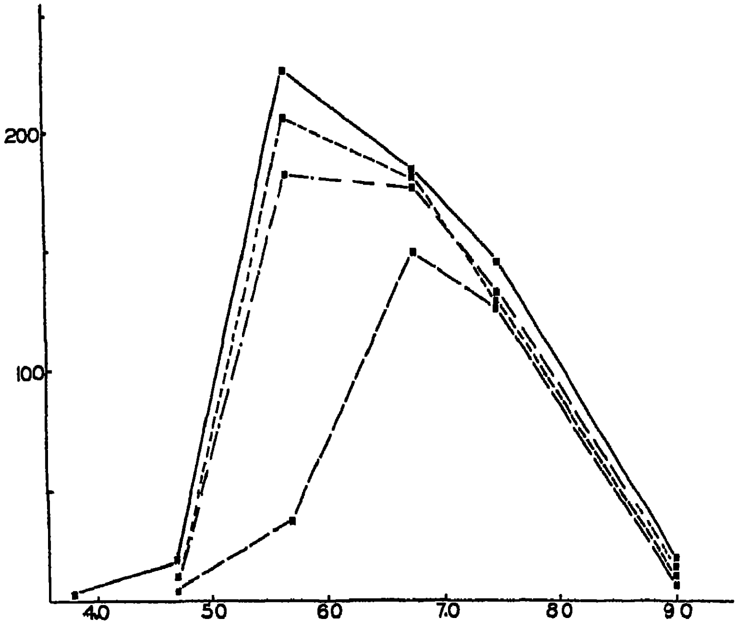


Fig. 2.—Series I*b*. Concentrations of ciliates in thousands per cubic centimeter are plotted against initial pH. Cultures are indicated the same as in Fig. 1 and indoleacetic acid (— · —) is added. The indole compounds have a concentration of 1:200,000.

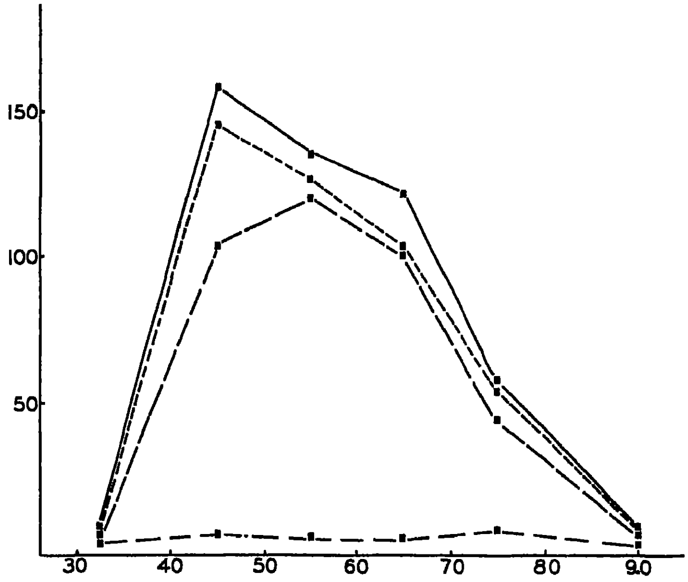


FIG. 3.—Series IIa. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. Cultures are designated the same as in Fig. 2. Concentration of indole compounds is 1 : 10,000.

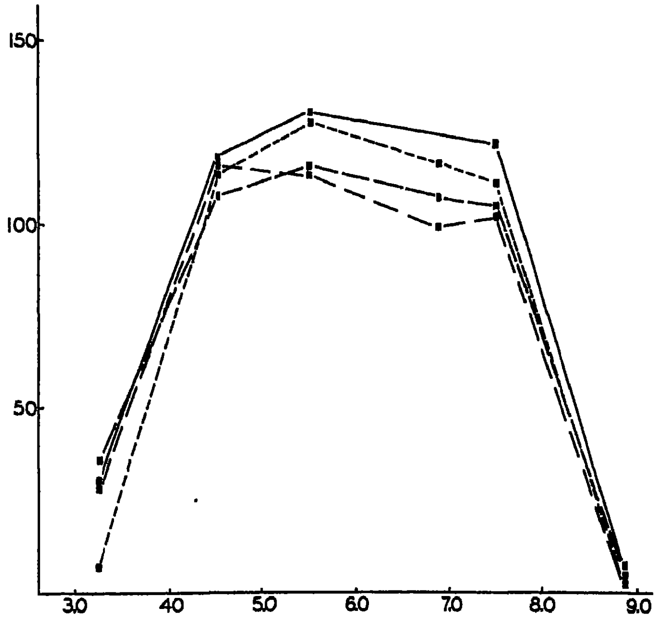


FIG. 4.—Series IIb. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. Cultures are indicated as in Fig. 2. Concentration of indole compounds is 1 : 200,000.

cent tryptone medium with initial pH as follows: 3.0, 4.5, 5.5, 6.6, 7.5, and 9.0. The initial count was 3,300 flagellates per cubic centimeter. No appreciable changes in pH had occurred after incubation for 6 days under constant artificial illumination.

The final counts (Fig. 5) showed acceleration of growth by indolebutyric acid over the entire pH range, and especially at pH 5.5. Indolepropionic acid produced significant acceleration at pH 5.5 but little or no effect at the other pH levels. Heteroauxin showed little or no effect over the pH range tested; as determined later (Series IV) this was due to the fact that a concentration of 1:200,000 is too high for this growth substance.

EFFECTS OF DIFFERENT CONCENTRATIONS OF PHYTOHORMONES

Series IV (*E. gracilis*).—Since acceleration of growth was pronounced only in the case of *E. gracilis*, experiments to determine the effects of different concentrations of indole compounds were limited to this species. The different indole compounds were added to flasks of 0.5 per cent tryptone medium in concentrations of 1:10,000, 1:100,000, 1:1,000,000, and 1:10,000,000. The initial pH in each flask was 5.6, and the initial count was 1,400 flagellates per cubic centimeter. The flask cultures were incubated under constant illumination for 9 days, 2.0-cc. samples being removed aseptically from each flask at 2-day intervals for determining the concentration of flagellates. The results (Fig. 6) show that growth is accelerated by indolepropionic acid in the three higher dilutions but not at 1:10,000. Indolebutyric acid showed marked acceleration of growth at dilutions of 1:1,000,000 and 1:10,000,000, but not in the highest concentration used. A decided acceleration was produced by indoleacetic acid in a concentration of 1:10,000,000; no effect was produced by 1:100,000, while growth was definitely retarded in a 1:10,000 dilution. No effort was made to plot the numbers of organisms beyond the seventh day of growth because counting became too difficult. However, counts on the ninth day with the "cytocrit"[2] indicated the same sort of relative growth as was observed on the seventh day. In all cases acceleration of growth, when it occurred, was obvious on the fourth day of growth. Other observations, not described here in detail, showed that the most effective concentration of indoleacetic acid lies between 1:1,000,000 and 1:10,000,000. These observations are in accord with the findings of Zimmerman and Wilcoxson (1935), who found indoleacetic acid to be five times as powerful as indolepropionic acid and indolebutyric acid in inducing responses in higher plants.

Series V (*E. gracilis*).—The same procedure was followed as in Series IV, except that the initial pH was 7.4 in all cases instead of 5.6. The results indicated that all three compounds were more effective in concentrations of 1:100,000–1:1,000,000 than in higher and lower dilutions in stimulating growth of *Euglena*. The accelerating effects were always slight, however, as compared with those observed at pH 5.6.

DISCUSSION

Since Went (1927) discovered a method for extracting plant hormones and demonstrating their quantitative effects on growth in *Avena* coleoptiles, much has been accomplished in identifying other substances which cause similar responses in higher plants. Zimmerman and Wilcoxon (1935) have shown that a large number of chemically diverse synthetic compounds cause responses in higher plants similar to those produced by auxins *a* and *b*. These synthetic compounds incite such striking effects as development of roots on leaves and stems, bending of stems, acceleration of growth, and epinasty

---

[2] A specially designed centrifuge tube used in estimating volume of packed protozoa in determining relative growth. This will be described in detail in a later publication.

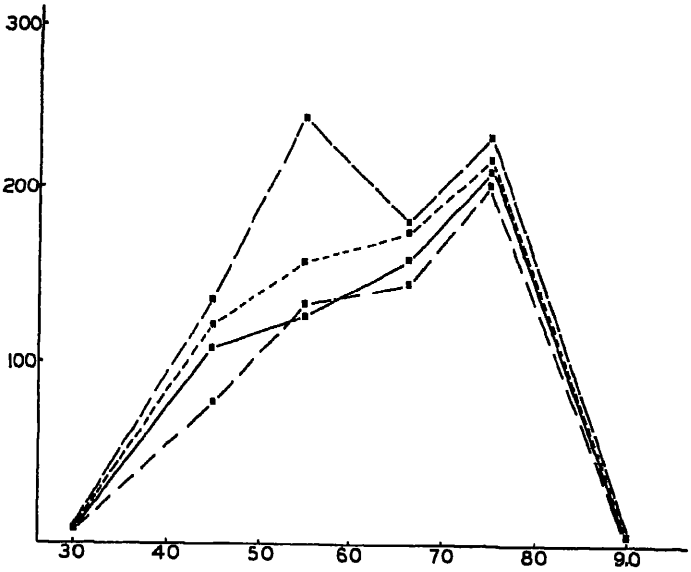


Fig. 5.—Series III. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. Cultures are designated the same as in Fig. 2 Concentration of indole compounds is 1:200,000.

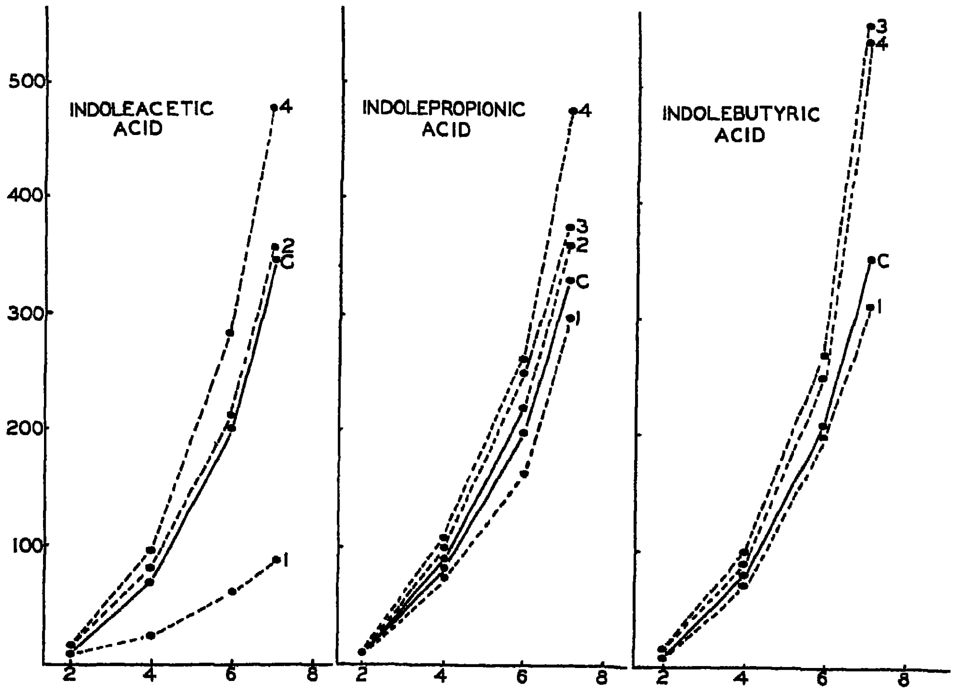


Fig. 6.—Series IV. Effect of different concentrations of phytohormones. Concentration of flagellates in thousands per cubic centimeter plotted against time in days. The dashed lines indicate cultures containing the indole compounds in concentrations indicated as follows: 1 = 1:10,000; 2 = 1:100,000; 3 = 1:1,000,000; 4 = 1:10,000,000. The solid lines are the controls in each case.

of leaves. Since such compounds produce profound changes in higher plants it might be expected that they would exert analogous effects on chlorophyll-bearing protozoa. Popoff (1933) demonstrated that low concentrations of growth substances extracted from coleoptiles and other parts of *Zea* seedlings accelerated cell division and excystment of *E. gracilis*. The purified indole compounds employed in this investigation also augmented multiplication of *E. gracilis*, indicating a marked influence on the rate of metabolism. The occurrence of autotrophic nutrition in *E. gracilis*, described by Dusi (1933) and earlier workers, has recently been questioned by Hutner (1936). If Hutner's findings can be corroborated, it may be inferred that *E. gracilis* shows a distinct tendency toward saprophytic nutrition. Nevertheless, this euglenoid flagellate still retains the property of utilizing the same substances which increase cell proliferation in higher plants.

It has been demonstrated that growth of lower plants, such as *Aspergillus niger*, is retarded by auxins, although such substances are produced in the tissues of the fungus (Boysen Jensen, 1932). Thus, auxins are found in fungi, yeasts, and bacteria, but usually fail to accelerate growth of the organism from which they are extracted. Whether or not *K. halli* elaborates such growth substances is not known, but it is obvious that growth was not accelerated by heteroauxin, indolepropionic, and indolebutyric acid. In this respect the flagellate resembles *A. niger*. The fact that *Khawkinea* is closely related to *Euglena*—the chief difference being the lack of chloroplasts in the former—reveals an apparent correlation between the presence of chlorophyll and the activity of plant hormones in Protozoa. That this flagellate is not photosynthetic has been demonstrated by Pringsheim (1921), who showed that it could not utilize ammonium compounds and single amino acids as sources of nitrogen. The change to saprophytism with loss of chlorophyll in this flagellate has been accompanied by a loss of the power to utilize growth substances which accelerate cell division of chlorophyll-bearing organisms.

These growth substances are found in animal cells and particularly in excretions such as urine. It is uncertain, however, that the auxins are synthesized by animal cells since they may possibly be derived from plants taken in as food. The available evidence indicates that these substances are not essential to growth of animal tissues. It has been shown, for instance, that growth of fibroblasts in tissue culture is not stimulated by auxins *a* and *b* (Kögl, Haagen Smit, and Tonnis, 1933). Likewise, the rate of metamorphosis of tadpoles is unaffected by auxin (Kögl, Haagen Smit, and Erxleben, 1933). Navez and Kropp (1934) failed to observe activity of the chromatophores of *Palaemonetes* when auxin was applied to the eyestalks. Greenberg and Schmidt (1936) observed no increase in growth of *Planaria maculata* when supplied with two different preparations of auxin. In view of the negative findings already obtained with animal cells, the present results with *Colpidium* were not surprising.

The effects of the indole compounds tested are interesting in comparison with results obtained in the case of pantothenic acid (Elliott, 1935*a*). With the latter, growth of *C. striatum* was markedly accelerated, but there was no effect on growth of the chlorophyll-bearing flagellate, *Haematococcus pluvialis*. With the indole compounds, the situation is reversed, no acceleration of growth being produced in the absence of chlorophyll.

The influence of hydrogen-ion concentration on the growth-promoting or inhibiting powers of the phytohormones showed a definite relationship. In those experiments where no acceleration was observed (Series I*a*, I*b*, II*a*, II*b*) the indole compounds were toxic throughout the entire pH range, but particularly so in the range of optimum growth (pH 4.5–6.0), with the exception of indoleacetic acid in Series I*a* and II*a* where it was extremely toxic at all pH levels. Above pH 7.0, even in concentrations of 1:10,000 of the indole compounds, toxic effects were much reduced over those on the acid side of neutrality. Furthermore, in Series III and IV acceleration of growth of *Euglena* occurred with the indole compounds at the points of optimum pH for growth (pH 4.5–6.0). It seems apparent, then, that if the indole compounds augment growth of a protozoan it must be done at the point of optimum growth for that organism. Substances which augment or inhibit growth of the three species of protozoa studied here do it at approximately the same pH level. This probably has some relationship with the ionization of the acids involved.

SUMMARY

Three plant hormones—3-indoleacetic acid (heteroauxin), β-3-indolepropionic acid, and γ-3-indolebutyric acid—were tested for their effect as growth stimulants on three species of Protozoa in pure cultures. Comparative studies were made with a chlorophyll-bearing flagellate, *E. gracilis*, a colorless euglenoid flagellate, *K. halli*, and a holotrichous ciliate, *C. striatum*. The compounds were tested over a wide pH range (3.0–9.0) and it was demonstrated that in the case of *Euglena* growth was markedly accelerated with all three indole compounds at pH 5.6 and a concentration of 1:1,000,000 and 1:10,000,000. No favorable effects were noted with either *Khawkinea* or *Colpidium*, and at certain concentrations of the plant hormones retardation of growth was observed.

LITERATURE CITED

Boysen Jensen, P. 1932. Über die Bildung und biologische Bedeutung des Wachstumsregulators bei *Aspergillus niger*. Biochem. Zeitschr., 250:270–80.

Dusi, H. 1933. Recherches sur la nutrition de quelques Euglenes. I. *Euglena gracilis*. Ann. l'Inst. Pasteur, 50:550–97.

Elliott, A. M. 1933. Isolation of *Colpidium striatum* Stokes in bacteria-free cultures and the relation of growth to pH of the medium. Biol. Bull., 65:45–56.

———. 1935*a*. The influence of pantothenic acid on growth of protozoa. Biol. Bull., 68:82–92.

———. 1935*b*. Effects of certain organic acids and protein derivatives on the growth of *Colpidium*. Archive f. Protistenk., 84:472–94.

Greenberg, L. D., and Schmidt, C. L. A. 1936. Studies on the properties of a growth-promoting factor for *Planaria maculata*. Jour. Exper. Zoöl., 73:375–92.

Hutner, S. H. 1936. The nutritional requirements of two species of *Euglena*. Archive f. Protistenk., 88:93–106.

Jahn, T. L., and McKibben, W. R. 1937. A colorless Euglenoid flagellate *Khawkinea halli*, n. gen., n. sp. Trans. Am. Micr. Soc., 54:48–54.

Kögl, F.; Haagen Smit, A. J.; and Erxleben, H. 1933. VII. Mitteilung. Studien über das Vorkommen von Auxinen im menschlichen und im tierischen Organismus. Hoppe-Seyl. Zeitschr. Phys. Chem., 220:137–61.

Kögl, F.; Haagen Smit, A. J.; and Tonnis, B. 1933. VIII. Mitteilung. Über das Vorkommen von Auxinen und von Wachstumsstoffen der "Bios"-Gruppe in Carcinomen. Hoppe-Seyl. Zeitschr. Phys. Chem., 220:162–72.

Navez, A. E., and Kropp, B. 1934. The growth promoting action of crustacean eye-stalk extract. Biol. Bull., 67:250–58.

Popoff, M. 1933. Über die pflanzlichen Auxine und ihre Wirkung auf Einzellige. Biol. Zentralbl., 53:661–68.

Pringsheim, E. G. 1921. Zur Physiologie der saprophytischen Flagellaten (*Polytoma*, *Astasia* und *Chilomonas*). Beitr. z. allg. Bot., 2:88–138.

Went, F. W. 1927. On growth-accelerating substances in the coleoptile of *Avena sativa*. Proc. K. Akad. Wetensch. Amsterdam, 30:10–19.

Zimmerman, P. W., and Wilcoxon, F. 1935. Several chemical growth substances which cause initiation of roots and other responses in plants. Contr. Boyce Thompson Inst. Plant Res., 7:209–29.

SOME EFFECTS OF HYPERTONIC SOLUTIONS UPON DEVELOPMENT AND OXYGEN CONSUMPTION[1]

(Twelve figures)

ARTHUR LOUIS SCHIPPER
Zoological Laboratory, State University of Iowa

INTRODUCTION[2]

CONSIDERABLE attention has been accorded the changes in water content of animal cells and tissues. Aquatic forms have served as experimental material for many of these investigations. The methods by which aquatic animals control the intake and outgo of water, together with the expenditure of energy necessary to maintain this water balance, has been thoroughly analyzed by Schlieper (1930, 1935). The role which proteins play in regulating the movements of water in living organisms, plus the oxygen consumption concerned in this regulation, has been emphasized by Jordan Lloyd (1933). Buxton (1932), investigating the humidity of the environment and terrestrial insects, and Mellanby (1935), examining the evaporation of water from insects, in extensive reviews have pointed out the physiological mechanisms governing water exchange.

Hypertonic solutions bring about alterations of the water content of animals and so produce changes in the weight (Adolph, 1936; Thompson and Bodine, 1936), volume (Kitching, 1934; Slifer, 1934; Thompson and Bodine, 1936), and oxygen consumption (Borei, 1935; Bodine, 1933, 1934; Thompson and Bodine, 1936).

Although the water content of insects under various conditions has been extensively studied, most observations have been on other than egg and embryonic stages. Bodine (1929) compared the water content and rate of oxygen consumption of developing *Melanoplus differentialis* eggs. The same author (1933, 1934) and, more recently, Thompson and Bodine (1936) have shown that hypertonic solutions depress the rate of oxygen consumption. Slifer (1934) reported that the resistance of postdiapause embryos to hypertonic solutions is dependent upon the concentration and the length of exposure. The purpose of this investigation has been to determine the effects of dehydration and dehydration-rehydration on cellular activity by noting viability, extent of development, and changes in the rate of oxygen consumption.

MATERIALS AND METHODS

Approximately 40,000 eggs of the grasshopper, *M. differentialis* (Thomas), have been used during the course of this investigation. The early stages (prediapause) have been designated as 1-, 5-, 10-, and 15-day eggs. After 17 or 18 days there is an interruption of development, diapause, which separates the pre- and postdiapause stages. Diapause is a naturally occurring period of inactivity concurrent with a low rate of oxygen consump-

---

[1] Aided by a grant from the Rockefeller Foundation for Research in Cellular Physiology.

[2] The writer wishes to express his appreciation to Professor J. H. Bodine for the manifest interest and many helpful suggestions rendered during the progress of this investigation.

tion and little change in water content. Eggs, collected on the day they were laid, were transferred to a constant-temperature chamber and kept there until the desired stage of development had been attained. (All eggs, control and experimental, were kept at 28° C.) Postdiapause eggs were obtained by subjecting diapause eggs to a low temperature (5° C.) for two months and then returning them to 28° C. The morphological stages have been described by Slifer (1932*a*, 1932*b*) and Slifer and King (1934). Bodine (1929) and Boell (1935) have determined the changes in the rate of oxygen intake during development.

Control eggs were kept on moist paper toweling while experimental ones were immersed in distilled water or in a balanced salt solution. Shell vials (1″ × 6″), containing 10 to 100 eggs in 25 cc. of solution, were suspended in a constant-temperature water bath. The solutions were changed daily.

The isotonic salt (modified Ringer's) solution was composed of 0.9 per cent NaCl and 0.02 per cent each of KCl and $CaCl_2$ and has been designated as N, the hypertonic solutions being designated accordingly (e.g., 5 N). Slifer (1934), using $NaHCO_3$ in addition to the above, found no adverse results when postdiapause embryos were placed in solutions ranging from 0.9 to 1.3 times the normal concentration.

Early in this investigation it was noted that the development of immersed eggs was retarded and eventually inhibited unless the solution containing the eggs was aerated. Aeration was effected by a continuous stream of air introduced into the solution through small-bore glass tubes; these aerating tubes were lined with a thin film of vaseline to prevent crystallization of the salts. Distributors, each with several taps, made possible the aeration of a large number of solutions simultaneously.

Modified Krogh microdifferential manometers, calibrated according to the method by Dixon (1934), were used in measuring changes in the rate of oxygen consumption. The number of eggs used in an experimental lot was dependent upon the stage of development attained, determinations being made with 20 prediapause, an equal number of diapause, and 10 postdiapause ones. The high rate of oxygen consumption of postdiapause eggs necessitated the use of fewer ones of this stage. Consecutive determinations for a single lot of eggs were made with the same manometer. Control eggs were placed in manometers with no solution while experimental eggs were placed in manometers containing 1 cc. of the solution to which the eggs had been exposed. The manometers were shaken while in the water bath to insure an equal distribution of oxygen dissolved in the solution. Each manometer cup was provided with a small well for KOH, 0.15 cc. of 2 per cent KOH being used to remove the carbon dioxide produced by the eggs.

EXPERIMENTAL RESULTS

DEVELOPMENT IN HYPERTONIC SOLUTIONS

The solutions employed in this investigation were 1, 2, 3, 4, 5, 7.5, 10, 15, and 20 N. One hundred 1-day eggs were placed in each of the solutions. Similar lots of eggs were placed in moist sand and in distilled water. The number of eggs developing was determined when the embryos were removed 20 days later (Fig. 1). More than 95 per cent of the eggs which had been in moist sand, distilled water, N, or 2 N solutions contained developing embryos; 85 per cent in 3 N, 50 per cent in 4 and 5 N, 25 per cent in 7.5 and 10 N and less than 10 per cent in 15 and 20 N. (The reader is referred to Table 1 for the development attained by these eggs.) Loss of water, as indicated by a loss of turgidity of the eggs, occurred in all hypertonic solutions. Some eggs in 5 N, and practically all in 15 and 20 N solutions, were flattened. This flattening was caused by the removal of water from the eggs by the hypertonic solutions. Solidification of the yolk, resulting in disintegration of the embryos, was probably responsible for the small percentage of developing eggs in the higher concentrations.

The effects of dehydration upon the extent of embryonic development were determined by placing 80 1-day eggs in each of the solutions. Embryos were removed and examined from some of the eggs 10 days later and at 5-day intervals thereafter (Table 1).

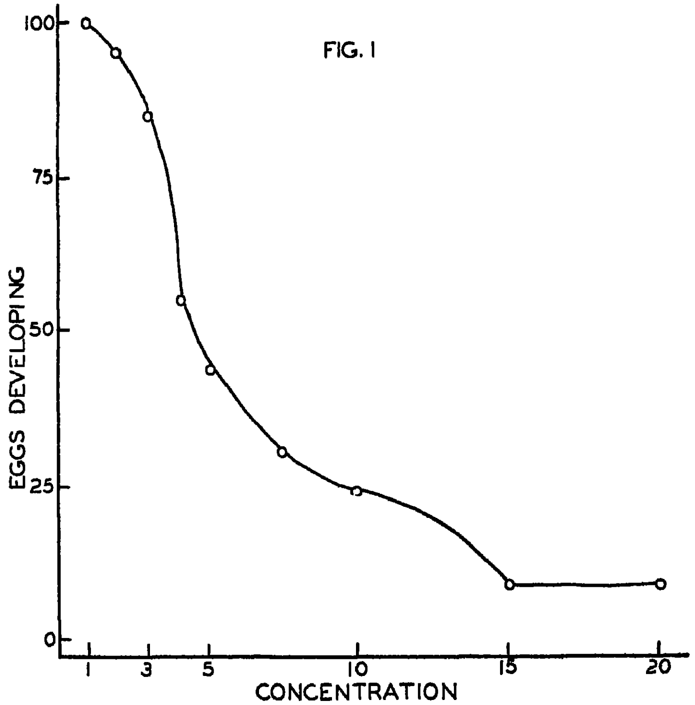


Fig. 1.—Eggs containing embryos after 20 days' exposure to solutions ranging from N to 20 N. Eggs were immersed on the day they were laid.

It is to be remembered that, with this retardation of growth, there was also a reduction in the number of eggs containing embryos (Fig. 1). This prolongation of the developmental period gradually merged into an inhibition of development; the stage at which cessation of growth occurred was dependent upon the hypertonicity of the solution. No embryos developed as far as the diapause stage in any of the hypertonic solutions.

Eighty postdiapause eggs were placed in each of the solutions on the day of removal from 5° C. A similar lot of eggs was kept on moist paper toweling. Hatching began in the control lot after 13 days and in N after 16 days. No hatching occurred in the hypertonic solutions, although many of the embryos had undergone considerable postdiapause development in concentrations ranging from 2 to 5 N. Beyond 5 N only a small number were able to initiate blastokinesis (Table 2). Reduction in size and disintegration of the embryos were frequently noted after exposures to hypertonic solutions.

Similar experiments were conducted with later stages of postdiapause eggs. Hatching was noted in 2 and 3 N if the postdiapause eggs were allowed to develop three days before being immersed in these solutions. If postdiapause eggs were allowed to develop six days before being dehydrated, hatching occurred in concentrations as great as 7.5 N. It is thus apparent that the eggs become more resistant to dehydration as postdiapause development continues.

TABLE 1[*]

EFFECT OF CONCENTRATION ON PREDIAPAUSE DEVELOPMENT

LENGTH OF EXPOSURE (DAYS)	CONCENTRATION								
	N	2 N	3 N	4 N	5 N	7.5 N	10 N	15 N	20 N
10	10[†]	9	9	8	8	7	7	4	4
15	15	11	11	10	10	9	8	4	4
20	D[‡]	13	13	12	11	10	9	4	4
25	D	15	14	12	12	11	9	4	4
30[§]	D	15	14	13	13	12	10	4	4

[*] One-day eggs were placed in solutions; the embryos of a few eggs were removed and examined at the intervals indicated.
[†] Stage of embryo as compared to control at 28° C.
[‡] Diapause.
[§] General inhibition after this period.

TABLE 2[*]

EFFECT OF CONCENTRATION ON POSTDIAPAUSE DEVELOPMENT

	CONCENTRATION								
	N	2 N	3 N	4 N	5 N	7.5 N	10 N	15 N	20 N
Percentage Initiating blastokinesis	90	63	66	77	70	35	15	27	11
Development attained[†]	*H*	*L*	*L*	*E*	*E*	*B*	*B*	*B*	*B*

[*] First-day postdiapause eggs were placed in solutions; the embryos were removed after 30 days' exposure.
[†] *H* = hatching; *L* = late yolk engulfment; *E* = early yolk engulfment; *B* = blastokinesis.

DEVELOPMENT FOLLOWING DEHYDRATION-REHYDRATION

The effects of hypertonic solutions were determined by dehydrating the eggs for a period of time and then transferring them to N. In this experiment five stages of development (1-, 5-, 10-, 15-day, and diapause) and five concentrations (5, 7.5, 10, 15, and 20 N) were used. The eggs were dehydrated 5, 10, 15, and 20 days in 5, 7.5, and 10 N; 4, 8, 12, and 16 days in 15 and 20 N. Following transfer to N some of the eggs burst owing to an excessive intake of water. This was most frequently noted with 5-day eggs and, to a lesser extent, with other stages. Such eggs, when examined, were found to contain no living embryos; the yolk presented a curdled appearance. Most of the embryos were removed 20 days after the eggs had been transferred to N and the number of developing embryos was determined (Table 3). A few eggs were allowed to remain, since it had been noted that the duration of the diapause condition was materially shortened by dehydration-rehydration. Hatching occurred in a few cases, being most frequent in those eggs which had been allowed to develop 15 days before being dehydrated. One-day eggs were quite resistant to 5 N except for long periods of immersion (20 days). The susceptibility increased with concentration and length of exposure. There was less recovery for 5-day eggs than for 1-day ones, the ability to overcome such treatment being determined by the concentration rather than by the length of dehydration. There was considerable recovery of 10- and 15-day eggs following dehydration by 5, 7.5, and 10 N although a marked susceptibility of these stages to 15 and 20 N was noted. Neither concentration nor length of immersion had much effect on the resistance of diapause eggs. It appears that the diapause eggs are able to retain water more tenaciously against osmotic forces than pre-diapause ones.

TABLE 3

RECOVERY OF DEHYDRATED EGGS FOLLOWING REHYDRATION

CONCENTRATION

5 N			7.5 N			10 N			15 N			20 N		
M*	E†	%‡	M	E	%	M	E	%	M	E	%	M	E	%
1	5	83	1	5	68	1	5	32	1	4	89	1	4	77
1	10	95	1	10	56	1	10	21	1	8	33	1	8	65
1	15	95	1	15	33	1	15	10	1	12	15	1	12	21
1	20	46	1	20	23	1	20	18	1	16	21	1	16	9
5	5	80	5	5	43	5	5	53	5	4	5	5	4	7
5	10	76	5	10	40	5	10	19	5	8	3	5	8	16
5	15	78	5	15	37	5	15	16	5	12	23	5	12	2
5	20	56	5	20	40	5	20	27	5	16	7	5	16	3
10	5	100	10	5	85	10	5	93	10	4	55	10	4	52
10	10	78	10	10	86	10	10	74	10	8	48	10	8	23
10	15	79	10	15	75	10	15	90	10	12	63	10	12	28
10	20	81	10	20	85	10	20	73	10	16	47	10	16	13
15	5	98	15	5	85	15	5	86	15	4	94	15	4	73
15	10	82	15	10	44	15	10	81	15	8	58	15	8	60
15	15	95	15	15	88	15	15	85	15	12	66	15	12	35
15	20	90	15	20	78	15	20	75	15	16	76	15	16	15
D§	5	100	D	5	63	D	5	97	D	4	90	D	4	81
D	10	98	D	10	52	D	10	78	D	8	90	D	8	90
D	15	97	D	15	82	D	15	73	D	12	88	D	12	90
D	20	100	D	20	87	D	20	82	D	16	91	D	16	73

* Morphological stage when dehydrated.

† Length of exposure to hypertonic solution.

‡ Recovery following transfer to N.

§ Diapause.

THE SECRETION OF THE CUTICLE IN HYPERTONIC SOLUTIONS

Twenty 1-day eggs were placed in each of the solutions and allowed to develop for 10 days. The contents of the eggs were then removed and the presence of the cuticular layers[3] was determined by chemical methods (Jahn, 1935). It was found impossible to prevent, or even delay to any extent, the secretion of either layer of the cuticule. The secretory function of the serosa cells is apparently little disturbed by hypertonic solutions despite the fact that the embryos are profoundly affected by them.

OXYGEN CONSUMPTION

The changes in the rate of oxygen consumption of developing *M. differentialis* eggs have been carefully measured by Boell (1935). In the present investigation somewhat higher rates were found, these being attributable to the higher temperature at which these experiments were conducted. Changes in the rate of oxygen intake of developing eggs, on moist paper toweling and immersed in distilled water and in N, are shown in Figures 2 (prediapause) and 3 (postdiapause). It will be noted that the control eggs possessed a higher rate of oxygen consumption than the immersed ones. The differences in the rates were probably caused by the slower rate of diffusion of oxygen through the liquid medium.

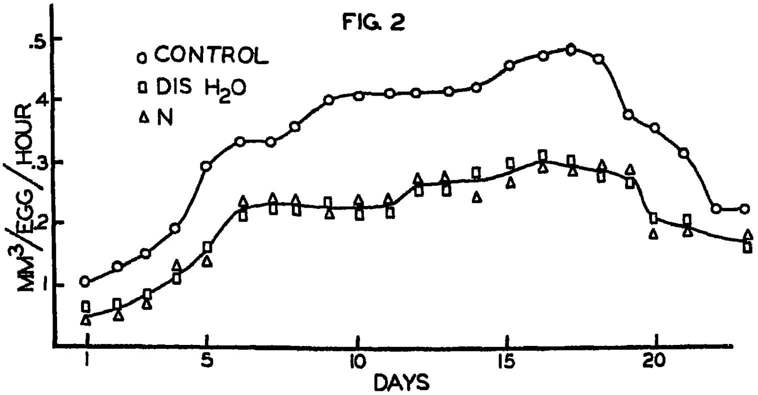


FIG. 2.—Changes in the rate of oxygen consumption of prediapause eggs

The effects of hypertonic solutions on the oxygen consumption of 1-, 5-, 10-, 15-day, diapause, first-, and third-day post-diapause eggs have been investigated. Typical results are presented in Figures 4–8. It was frequently noted that the rate of oxygen consumption of a given stage of development was similarly affected by two or more concentrations. In such instances one concentration, together with the results produced by that concentration, was chosen to represent all those producing like results. Generally speaking, for the prediapause and diapause stages, there was but little difference in the rates of oxygen intake of these eggs in concentrations ranging from 3 to 10 N; likewise 15 and 20 N produced strikingly similar results (Figs. 4–6). A greater difference in the rates of oxygen consumption was noted for postdiapause eggs in hypertonic solutions (Figs. 7 and 8). The rate of oxygen consumption of eggs in the various hypertonic solutions was largely dependent upon the stage at which dehydration was started. These differences were most marked in those eggs which previously had had a high rate of oxygen intake (Fig. 8); likewise there was but little difference if the rate of oxygen consumption previous to immersion was low (Fig. 6).

---

[3] The histology of these layers has been described by Slifer (1937).

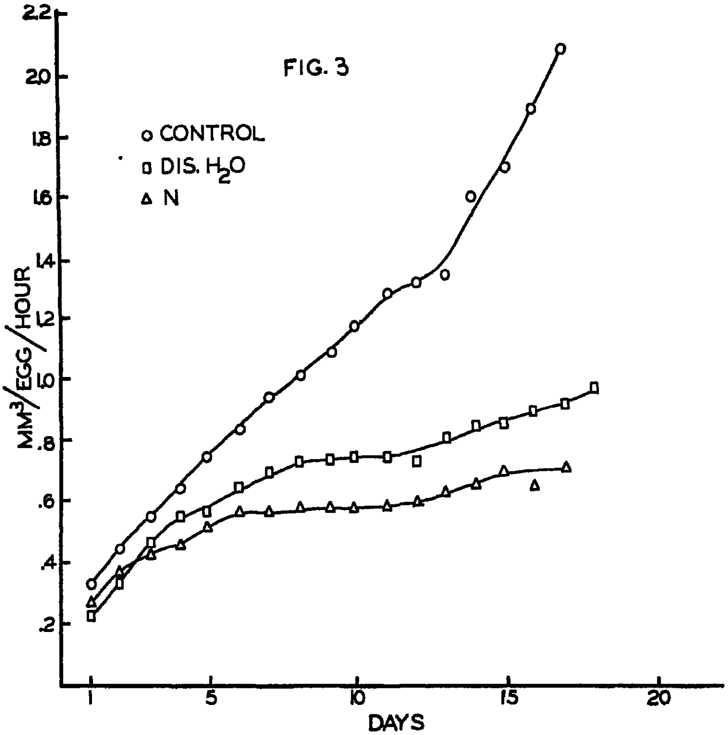
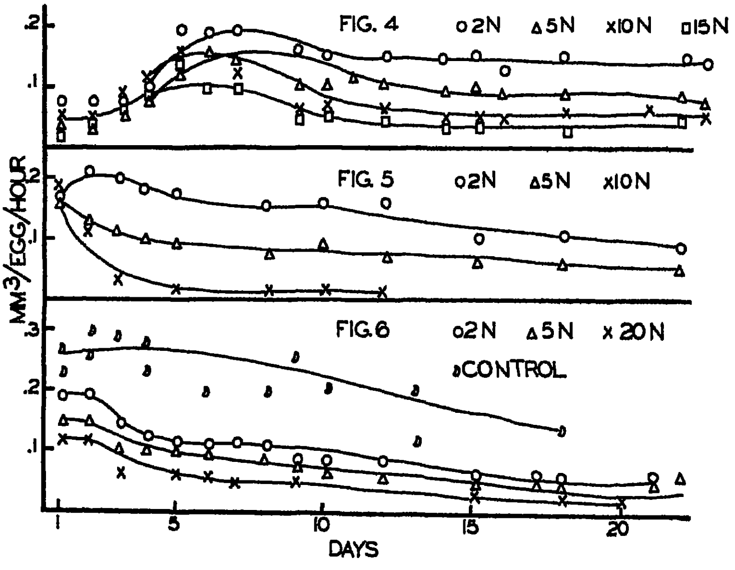
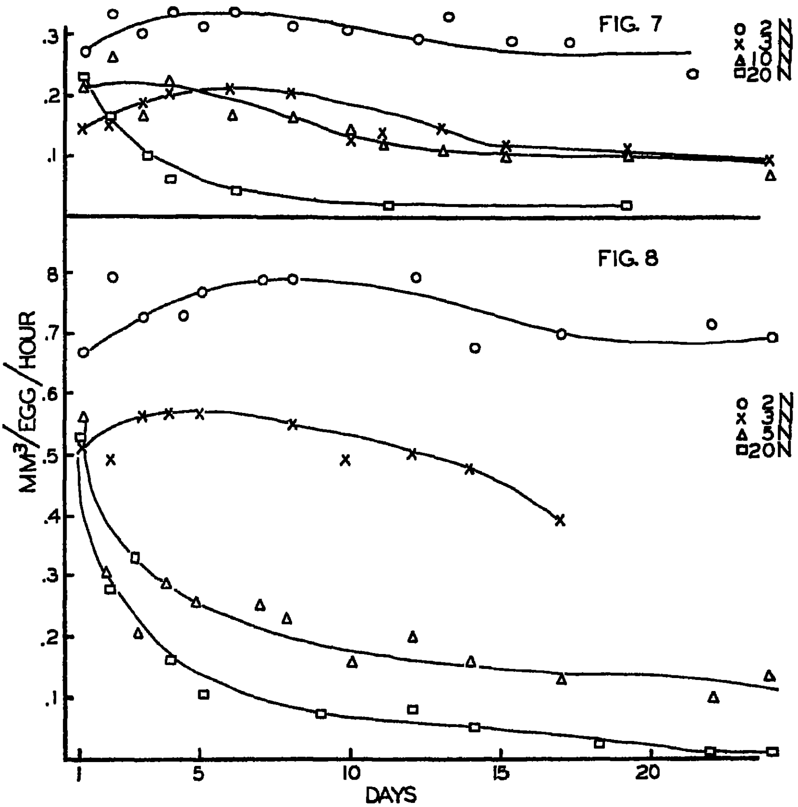


Fig. 3.—Changes in the rate of oxygen consumption of postdiapause eggs



Figs. 4–6.—Figure 4, changes in the rate of oxygen consumption of 1-day eggs subsequent to immersion in hypertonic solutions. Figure 5, changes in the rate of oxygen consumption of 5-day eggs subsequent to immersion in hypertonic solutions. Figure 6, changes in the rate of oxygen consumption of diapause eggs subsequent to immersion in hypertonic solutions.

When 1-day eggs were placed in hypertonic solutions there was an initial increase in the rate of oxygen consumption, the peak being on the fifth and sixth days of exposure (Fig. 4); subsequent to the peak there was a decrease. As has been noted earlier in this investigation, embryos were formed in all concentrations, but further development depended upon the hypertonicity of the solution (Table 1). If the delay in development is compared with the reduction in the rate of oxygen consumption (Fig. 4), it will be noted that those solutions which cause the greater retardation also effect a corresponding depression of the oxygen intake.



FIGS. 7–8.—Figure 7, changes in the rate of oxygen consumption of first-day postdiapause eggs subsequent to immersion in hypertonic solutions. Figure 8, changes in the rate of oxygen consumption of third-day postdiapause eggs subsequent to immersion in hypertonic solutions.

When any of the following stages, 5-, 10-, 15-day, and diapause, were placed in hypertonic solutions there was an immediate decrease in the rate of oxygen consumption, the rate later tending to become constant. The changes in the rate of oxygen consumption of 5-day and diapause eggs are shown in Figures 5 and 6. The amount and rate of the decrease in oxygen intake were dependent upon the physiological stage of the cells at the time when the eggs were subjected to the hypertonic solutions. This is well illustrated by the effects of 10 N on 5-day eggs (Fig. 5) and of 20 N on diapause (Fig. 6). The cells of 5-day eggs are in a period of active differentiation while those of the diapause eggs are in a state of quiescence owing to a developmental block. There was a marked decrease in the rate of oxygen consumption of 5-day eggs in 10 N although only a slight one was noted for diapause eggs in 20 N. The effects of hypertonic solutions on the amount of oxygen consumed by 10- and 15-day eggs were intermediate between those found for 5-day and diapause eggs. A general retardation of development of the prediapause eggs in hypertonic solutions was noted at the end of the experiment.

The rate of oxygen consumption of postdiapause eggs in hypertonic solutions varied considerably, depending upon the concentration of the solution and the stage of development (Figs. 7 and 8). There was a marked difference in the rates of oxygen intake of postdiapause, diapause, and prediapause eggs in hypertonic solutions. This is to be noted when the rates of diapause eggs in 2, 5, and 20 N, as shown in Figure 6, and the rates of postdiapause eggs in 2, 3, and 20 N, as shown in Figures 7 and 8, are compared. It is to be remembered that postdiapause eggs have much higher rates of oxygen consumption than diapause ones. The differences in the rates of oxygen intake of postdiapause eggs in hypertonic solutions become much greater when later stages are dehydrated. The differences between the rates of oxygen consumption of postdiapause eggs in 2 and in 3 N is nearly as great as the difference between the rates of similar eggs in 3 and in 20 N. First-day postdiapause eggs in 3 and in 10 N consume oxygen at similar rates (Fig. 7) while 3-day postdiapause eggs in 3 and in 5 N utilize oxygen at widely separated rates (Fig. 8). The cause of these differences may be found in the increased size, activity, and water content of the older embryos. Any marked reduction of the water content, thus affecting the metabolic activity, would tend to depress the rate of oxygen consumption.

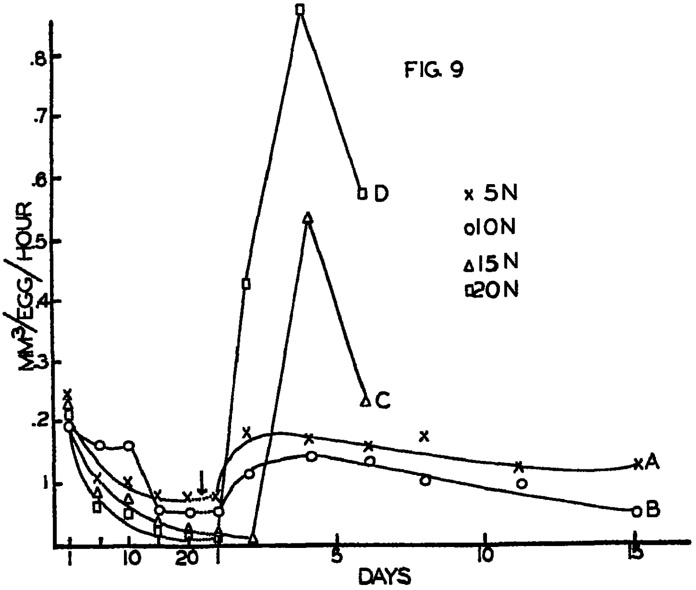
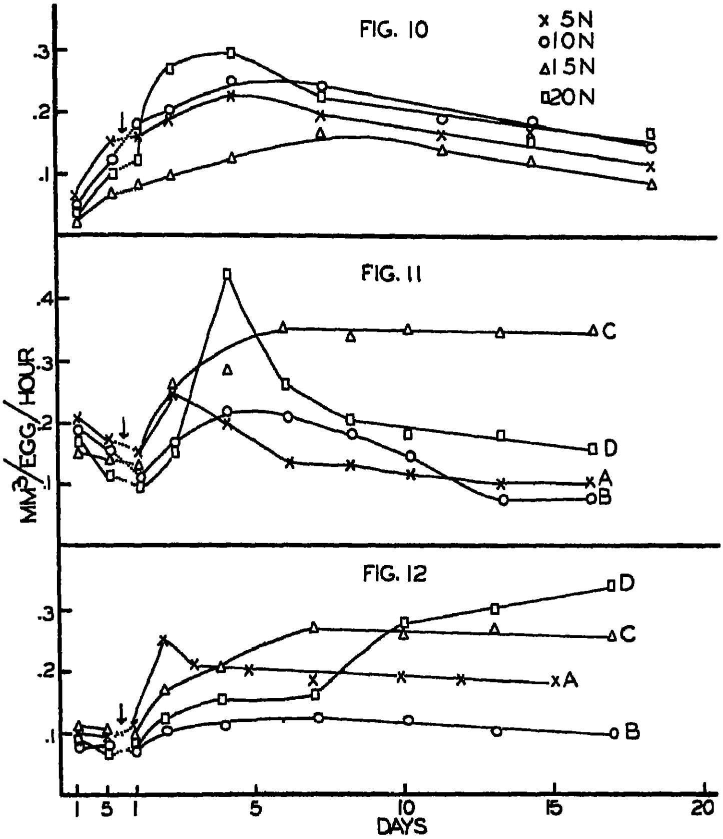


FIG. 9.—Changes in the rate of oxygen consumption of 15-day eggs immersed in hypertonic solutions for 20 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale.

THE EFFECTS OF DEHYDRATION-REHYDRATION ON OXYGEN CONSUMPTION

The recovery of dehydrated prediapause and diapause eggs after rehydration has been noted earlier in this report (Table 3). The present experiment was conducted in an attempt to correlate the changes in susceptibility with the changes in the rate of oxygen consumption. Five stages of development (1-, 5-, 10-, 15-day, and diapause) and four concentrations (5, 10, 15, and 20 N) were used. The lengths of exposure were 5, 10, 15, and 20 days. Following exposure to hypertonic solutions, the eggs were transferred to N. The responses of the dehydrated eggs to rehydration were dependent upon (1) the degree of dehydration, (2) the stage of development when dehydrated, and (3) the effect on diapause. Typical results have been chosen to illustrate these points.



Figs. 10–12.—Figure 10, changes in the rate of oxygen consumption of 1-day eggs immersed in hypertonic solutions for 5 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale. Figure 11, changes in the rate of oxygen consumption of 10-day eggs immersed in hypertonic solutions for 5 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale. Symbols as in Figure 10. Figure 12, changes in the rate of oxygen consumption of diapause eggs immersed in hypertonic solutions for 5 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale. Symbols as in Figure 10.

The degree of dehydration was dependent upon the hypertonicity of the solution and the length of exposure (see Thompson and Bodine, 1936). There is, moreover, a corresponding depression in the rate of oxygen consumption (Figs. 4–8). If the rate of oxygen intake of eggs, while in a hypertonic solution, fell below 0.04 $mm.^3$/egg/hour (indicative of a great water loss) death of the eggs usually resulted. Such eggs, when transferred to N, consumed oxygen at a much higher rate (Fig. 9*C*, *D*). However, if the rate of oxygen consumption did not drop to such a low level, transfer to N was followed by an increase, later giving way to a decrease (Fig. 9*A*, *B*).

Rehydration of dehydrated eggs always elicited an increase in the rate of oxygen intake. In most instances this increase was followed by a decrease, the amount and rate of which were dependent upon the stage of development attained by the eggs prior to dehydration. If 1- and 5-day eggs were placed in hypertonic solutions and later transferred to N, then the decrease in the rate of oxygen consumption which followed the initial increase was quite marked (Figs. 10 and 11*A*, *B*, *D*). Such a decrease was slight or entirely absent if more advanced stages were used (Figs. 9*A*, *B* and 12*A*, *B*). In such cases the rate of oxygen intake eventually became constant. When the eggs were examined at the end of the experiment it was noted that most of the embryos had developed to the diapause condition.

The abbreviation or elimination of diapause by dehydration-rehydration noticeably affected the rate of oxygen consumption. Following rehydration there was a rapid intake of oxygen, the increase later becoming more gradual (Figs. 11*C* and 12*C*, *D*). Upon examination of the eggs at the end of the experiment, the developing postdiapause embryos responsible for the higher rates of oxygen consumption were noted.

DISCUSSION

The development of the embryos of *M. differentialis* is retarded when the eggs are dehydrated, the rate of growth being inversely related to the hypertonicity of the solution. Ludwig (1936) found that the duration of the pupal stage of *Popillia japonica* was increased by desiccation, this increase being inversely proportional to the water content of the pupae. The prolongation of development of grasshopper eggs gradually merges into a cessation of growth, this depending upon the embryonic stage and the concentration of the solution in which the eggs are immersed. Some embryonic growth is manifested in each of the hypertonic solutions (Table 1) although the percentage of embryos appearing in each concentration may differ (Fig. 1). Development in hypertonic solutions appears to be conditioned by the amount of water in the egg; cessation of growth occurs when the water content of the egg is lowered below a level sufficient to sustain further development. Bodine (1929) found that there was a corresponding increase in the water content of the egg as development progressed. From this it follows that a concentration which would dehydrate an egg rapidly would also effect a cessation of development more quickly. If the hypertonicity were less, development would continue at a retarded rate until there was an insufficient amount of water in the eggs to sustain further growth.

It is interesting to note that eggs of *Anisoplia austriaca* are able to extract water from 4 per cent NaCl, 2 per cent KNO_3, and 2 per cent $BaCl_2$ (Kerenski, 1930). It is not likely that eggs of *M. differentialis* are able to remove water from a hypertonic solution since a loss of turgidity has been noted following such treatment. Thompson and Bodine (1936) found that the postdiapause eggs of the grasshopper lose water to hypertonic solutions, the dehydration being rapid at first and then more gradual.

Recovery of dehydrated eggs, following rehydration, is dependent upon the physiological state of the egg when dehydrated and the concentration of the salt solution. The resistance of grasshopper eggs to dehydration has been determined, the order being (maximum to minimum): diapause, 15-, 10-, 1-, and 5-day eggs. Similar results have been found following irradiation (Evans, 1935, 1936). One of the probable factors concerned with the change in susceptibility is the activity of the cells. Diapause is a state of quiescence in which cellular activity is at a minimum. Slifer (1931) has shown that there is little or no mitotic activity in diapause embryos although such activity is extensive in prediapause ones. Slifer did not extend her investigation to postdiapause embryos but it is certain that such embryos would contain many dividing cells. Active differentiation is taking place with the formation of the embryo. It is during this period of active differentiation that the eggs are most susceptible to hypertonic solutions.

Ludwig (1936) noted that larvae of *P. japonica* could withstand a greater degree of dehydration when such water loss was not rapid. Although the eggs were not weighed in the present investigation, it is possible to compare the effects of different concentrations. In general the recovery is greater even after long exposures (20 days) to 5, 7.5, and 10 N than after short exposures (4 days) to 15 and 20 N.

Thompson and Bodine (1936) found that postdiapause eggs were usually killed after a water loss exceeding 2.5 mg. Such eggs, upon transfer to isotonic solution, became very swollen, the egg membranes frequently being ruptured. In the present investigation swelling and bursting have also been noted following rehydration of dehydrated eggs, the rupturing of the egg membranes occurring most frequently when 5-day eggs were so treated. Since the cuticle is secreted even when the eggs are in hypertonic solutions, this effect on 5-day eggs does not appear to be caused by the condition of the cuticular layers unless dehydration affects the thickness or some other characteristic of the white layer. It is not likely that the secretion of the white layer has progressed far in 5-day eggs. Secretion of this layer begins on the seventh day at 25° C. (Slifer, 1937).

In considering the effects of hypertonic solutions it is to be noted that eggs in various morphological stages behave differently. It is doubtful that these results are attributable entirely to the physiological state of the eggs. The effect of these solutions on the yolk is also to be considered since the yolk plays an important role in the environment of the embryo. The yolk/embryo ratio is extremely large in early prediapause, becoming very small in late postdiapause. Dehydration of the eggs might render the yolk cells incapable of liquefying the yolk and thus preventing its assimilation by the embryo. In such cases loss of water would tend to inhibit the development of the embryo.

The extent of development of postdiapause eggs in hypertonic solutions appears to be dependent upon the amount of water present. Adolph (1933) suggests that muscular contraction involves translocation of water. It is, therefore, quite possible that the degree of dehydration would tend to regulate the blastokinetic process. The findings, concerning the development of postdiapause eggs in hypertonic solutions, would serve to add credence to the above argument.

Previously only a reduction in the rate of oxygen consumption has been noted when eggs of *M. differentialis* were dehydrated (Bodine, 1933, 1934; Thompson and Bodine, 1936). The results of this investigation agree with the above findings where the data are comparable; however, an increase occurs in the oxygen intake of both 1-day eggs in all concentrations and of later postdiapause eggs in 2 and 3 N solutions. Bodine (1933) has suggested that the effects of hypertonic solutions are largely osmotic since NaCl solutions, of the same osmotic pressure as the hypertonic balanced salt solutions, produced similar depressions in the rate of oxygen consumption.

Rehydration of dehydrated eggs is always followed by an increase in the rate of oxygen intake. The amount of oxygen consumed is dependent upon whether or not the effects of dehydration are reversible. Eggs which have been killed by dehydration usually possess a very high rate of oxygen consumption when rehydrated, this probably being caused by the actions of molds and bacteria. Irreversible results occur after the eggs have lost an excessive amount of water (see Thompson and Bodine, 1936). Depression of the rate of oxygen consumption by hypertonic solutions may be used as an index to irreversibility (Bodine, 1933). Any reduction of the water content of prediapause eggs, which lowers the rate of oxygen intake to 0.04 $mm.^3$/egg/hour, seems to be a sufficient water loss to prevent recovery.

SUMMARY

Retardation is noted when eggs are dehydrated, development continuing as long as the eggs contain a sufficient amount of water to sustain growth.

Five-day eggs are the most susceptible to hypertonic solutions, diapause the most resistant.

Dehydration-rehydration may serve to shorten or eliminate the developmental block.

The secretion of the yellow and white layers of the cuticle is not prevented by hypertonic solutions.

The rate of oxygen consumption of 1-day eggs, in hypertonic solutions, increases for 5 days before a decrease is noted. Dehydration of other prediapause stages results in an immediate decrease in the rate of oxygen intake.

The differences in the rates of oxygen consumption of eggs, in the various hypertonic solutions, are largely dependent upon the initial stage of the eggs.

Rehydration of dehydrated eggs results in an increase in the rate of oxygen intake, this increase usually being followed by a decrease.

LITERATURE CITED

ADOLPH, E. F. 1933. The metabolism and distribution of water in body and tissues. Physiol. Rev., 13: 336–71.

———. 1936. Differential permeability to water and osmotic changes in the marine worm *Phascolosoma*. Jour. Cell. and Comp. Physiol., 9:117–35.

BODINE, J. H. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., 2:459–82.

———. 1933. The effects of hypertonic solutions on the oxygen consumption of a developing egg (Orthoptera). *Ibid.*, 6:150–58.

-. 1934. To what extent is the developmental block dependent upon the metabolic activity of the embryonic cell? Proc. Nat. Acad. Sci., 20: 640–44.

BOELL, E. J. 1935. Respiratory quotients during embryonic development (Orthoptera). Jour. Cell. and Comp. Physiol., 6:369–85.

BOREI, H. 1935. Über die Einwirkung des Salzgehaltes auf den O_2-Verbrauch des Echinodermeneies. Z. f. Morph. u. Ökol. d. Tiere., 30: 97–98.

BUXTON, P. A. 1932. Terrestrial insects and the humidity of the environment. Biol. Rev., 7:275–320.

DIXON, M. 1934. Manometric methods as applied to the measurement of cell respiration and other processes. Cambridge, Eng.: University Press.

EVANS, T. C. 1935. Variations in susceptibility to x-rays of *Melanoplus differentialis* eggs during development. Physiol. Zoöl., 8:521–29.

———. 1936. Qualitative and quantitative changes in radiosensitivity of grasshopper eggs during early development. *Ibid.*, 9:443–54.

JAHN, T. L. 1935. Nature and permeability of grasshopper egg membranes. II. Chemical composition of membranes. Proc. Soc Exp. Biol. Med., 33:159–63,

JORDAN LLOYD, D. 1933. The movements of water in living organisms. Biol. Rev., 8:463–81.

KERENSKI, J. 1930. Beobachtungen uber die Entwicklung der Eier von *Anisoplia austriaca* Reitt. Z. f. angew. Ent, 16: 178–88. (Cited by Buxton, 1932.)

KITCHING, J. A. 1934. The physiology of contractile vacuoles. I. Osmotic relations. Jour. Exper. Biol., 11:364–81.

LUDWIG, D. 1936. The effect of desiccation on survival and metamorphosis of the Japanese beetle (*Popillia japonica* Newman). Physiol. Zool., 9:27–42.

MELLANBY, K. 1935. The evaporation of water from insects Biol. Rev., 10:317–33.

SCHLIEPER, C. 1930. Die Osmoregulation wasserlebender Tiere *Ibid.*, 5:309–56.

———. 1935. Neuere Ergebnisse und Probleme aus dem Gebiet der Osmoregulation wasserlebender Tiere. *Ibid.*, 10:334–60.

SLIFER, E. H. 1931. Insect development. II. Mitotic activity in the grasshopper embryo. Jour. Morph. and Physiol., 51:613–18.

———. 1932*a*. Insect development. III. Blastokinesis in the living grasshopper egg. Biol. Zentralbl., 52: 223–29.

———. 1932*b*. Insect development. IV. External morphology of grasshopper embryos of known age and with a known temperature history. J. Morph., 53: 1–21.

———. 1934. Insect development. VI. The behavior of grasshopper embryos in anisotonic, balanced salt solutions. J. Exp. Zool., 67: 137–57.

———. 1937. The origin and fate of the membranes surrounding the grasshopper egg: together with some experiments on the source of the hatching enzyme. Quart. J. Micr. Sci., 79: 493–506.

SLIFER, E. H., and KING, R. L. 1934. Insect development. VII. Early stages in the development of, grasshopper eggs of known age and with a known temperature history. Jour. Morph. and Physiol., 56:593–98.

THOMPSON, V., and BODINE, J. H. 1936. Oxygen consumption and the rate of dehydration of grasshopper eggs (Orthoptera). Physiol. Zool., 9:455–70.

A STUDY OF THE MECHANISM OF CYANIDE INHIBITION: I. EFFECT OF CONCENTRATION ON THE EGG OF MELANOPLUS DIFFERENTIALIS[1]

(Seven figures)

W. A. ROBBIE, E. J. BOELL, AND J. H. BODINE
Zoölogical Laboratory, State University of Iowa

RESULTS obtained from studies of the depressing action of cyanide on the respiration of animal cells and tissues have been used by Warburg (1928) in support of a theory that respiration is due primarily to an activation of oxygen by an iron-containing enzyme. A complete discussion of this subject is found in Warburg's original works (1928) and in reviews by Dixon (1929) and Meldrum (1934) to which the reader is referred.

The present experiments have been designed primarily to determine the variations in cyanide sensitivity of the respiratory mechanism of grasshopper eggs using a manometric technique relatively free from difficulties ordinarily inherent in such work.

MATERIAL AND METHODS

Eggs of the common grasshopper, *Melanoplus differentialis*, with a known temperature history and of a known chronological and morphological age, were used throughout. Eggs studied during the prediapause and diapause periods of development were kept at 25° C. in the pods in which they were laid until they were desired for use. Those employed in the postdiapause determinations were kept in the pods at 25° C. until well into the diapause period at which time they were placed at 10° C. to break the diapause block. After two months or more at this temperature they were returned to 25° C. and resumed development as postdiapause eggs. Morphological ages of embryos were checked periodically.[2]

Measurements of respiration were made with Barcroft-Warburg manometers at 25° C. The number of eggs used in each determination varied from 35 to 200 depending upon the stage of development.

In cyanide experiments carried out by many of the earlier investigators the tissues or cells to be exposed were placed in a manometer flask with unneutralized KCN, using KOH as the CO_2 absorbing medium in the center well. Later Dixon and Elliott (1929) neutralized the cyanide to eliminate pH effects. With either of these techniques HCN gas distilled over from the suspension medium to the KOH in the center well, resulting in a rapid decrease of active HCN in the system. To avoid this difficulty some investigators replaced the KOH center well solution with one of KCN or NaCN sufficiently concentrated to absorb all of the CO_2 produced, and, because of its positive HCN ten-

---

[1] Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

[2] In this paper development is always considered as days at 25° C. since the morphological stages of development have been described by Slifer (1932) in such terms. For details concerning diapause and the physiology of blocked and developing eggs see Slifer (1932), Bodine (1932), Bodine and Boell (1934), and Boell (1935).

sion, to prevent any absorption of HCN from the suspension medium (Schmitt and Schmitt, 1931). Since this practice sometimes resulted in an increased HCN content of the fluid in which the cells or tissues were immersed, van Heyningen (1935), following Krebs (1935), employed as the center-well absorbing medium a mixture of KCN and KOH of such proportions that the HCN tension was equal to that of the immersion fluid.

To check the efficiency of these methods of exposure to cyanide for the present investigations, three sets of experiments were carried out, the results of which are shown graphically in Figure 1. In Experiments A and B one hundred 18-day prediapause eggs were run in each manometer with 3 cc. of 0.0025M KCN as the experimental fluid; the center-well solutions used are indicated in the explanation of the figure. At the end of a 3-hour exposure to the KCN the manometer flasks in Experiment A were removed, the eggs washed, returned to the flasks with water as the suspension medium, and replaced in the bath. The flasks in Experiment B were not removed, the eggs remaining continuously in cyanide.

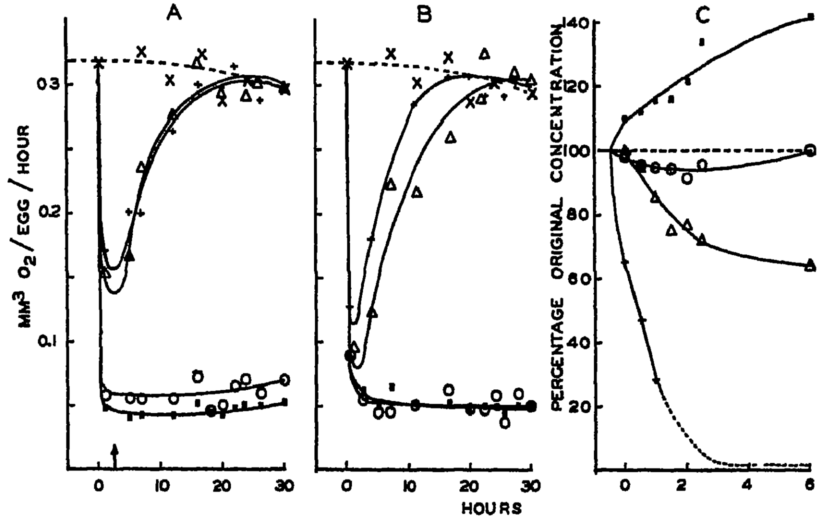


FIG. 1.—Comparison of techniques in the use of cyanide. (*A*) Arrow indicates the time at which the eggs were removed from the cyanide, washed, and returned to the manometers. (*B*) Eggs left in cyanide solutions continuously. (*C*) Variations in cyanide concentration in manometers containing same solutions as *A* and *B* but no eggs. Initial concentration of KCN = 0.0025M. × = control; eggs in water, 10 per cent KOH in inset. △ = unneutralized KCN, 10 per cent KOH in inset. + = KCN neutralized in pH. 7.0, 10 per cent KOH in inset. ■ = 0.0025M neutralized KCN as experimental fluid, 0.5M KCN in inset. ○ = 0.0025M neutralized KCN as experimental fluid, balanced KCN-KOH mixture in inset.

Chart C of Figure 1 shows the results of an analysis of cyanide concentration existing in the experimental fluid at various times after the start of the exposure. In the determination of each of the curves solutions similar to those used in A and B but containing no eggs were put in seven manometer flasks. These were then placed in the 25° C. water bath and shaken just as in Experiments A and B. At ½-hour intervals a manometer was removed and duplicate samples of the experimental fluid in the flask were analyzed by the method of Smith (1929).

An inspection of the curves shows that those eggs left continuously in cyanide solutions with KOH in the center well (Fig. 1*B*) recover about as quickly as those which are removed and washed at the end of a 3-hour exposure (Fig. 1*A*). This is apparently due to a diminished concentration resulting from the distillation of HCN to the KOH in the center well. With neutralized KCN less than 30 per cent of the cyanide originally present remains after the experiment has been run for an hour (Fig. 1*C*). The same graph shows that the cyanide concentration in the manometer flask containing strong unbalanced KCN center-well solution is 120 per cent of the original value at the end of 2 hours and 140 per cent within 6 hours. With the balanced KCN-KOH center-well solution recommended by Krebs and van Heyningen for 0.0025M KCN, the concentration at the end of the 6-hour period is 100 per cent of the original value, and during this time it does not vary more than 10 per cent (Fig. 1*C*).

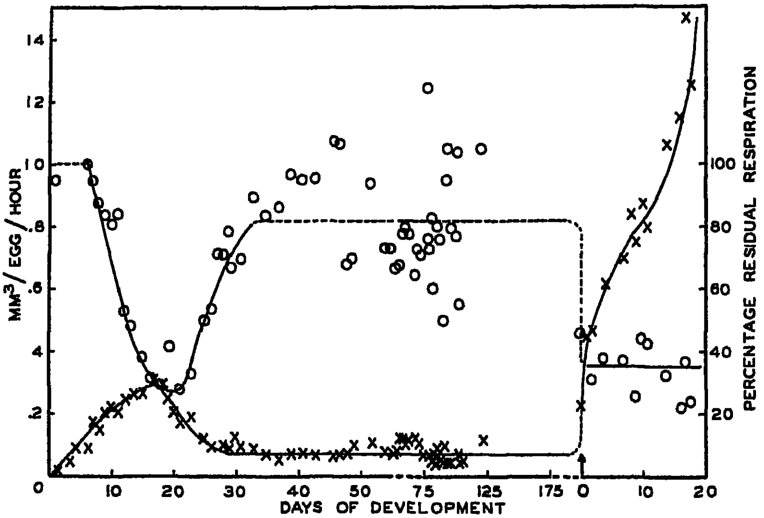


Fig. 2.—Variations in cyanide sensitivity during embryonic development. Left-hand ordinate represents O_2 consumed by control eggs (X). Right-hand ordinate shows percentage residual respiration of eggs treated for three hours with 0.001M KCN (O). Abscissa denotes days of development at 25° C. Prediapause period from 0 to 20 days; diapause from 21 days to arrow (diapause here terminated artificially as indicated in text); postdiapause period from 0 to 17–19 days (hatching). Each symbol represents an average value for the day indicated. Chart includes more than 250 experiments.

Difference in the recovery of eggs treated according to the technique of Schmitt and that of Krebs may indicate that the use of strong unbalanced KCN center-well solutions for exposures as short as 3 hours is an unsafe procedure.

The balanced center-well solutions recommended by Krebs (1935) have been used in all of the experiments described in this paper except in the preliminary ones indicated above. All of the work was carried out at a temperature of 25° C. and the exposures to cyanide, unless otherwise indicated, have been for periods of 3 hours.

PREDIAPAUSE PERIOD

Residual respiration of the egg in 0.001M KCN (Fig. 2) varies from 100 to 80 per cent of the control value during the sixth to the tenth day of development, but on the twentieth day is only 30 per cent of the original level.[3] This increasing sensitivity to the action of cyanide as the egg grows older may be correlated with the increasing size of the embryo and the changes in the yolk cells during the prediapause period.

Following the twentieth day the cyanide sensitivity decreases to the diapause level. Figure 2 also indicates that the prediapause change in sensitivity is perhaps related to the course of normal O_2 uptake for the eggs. As the normal respiration of the egg increases with development the depressing effect of cyanide becomes more pronounced. Correspondingly, when the normal respiration decreases with the onset of diapause, the action of cyanide becomes less noticeable. This relationship of sensitivity and respiration level is shown also by Figure 5. Buchanan (1926) has similarly found that with *Planaria* a relatively greater depression is produced by cyanide as the rate of oxygen consumption increases.

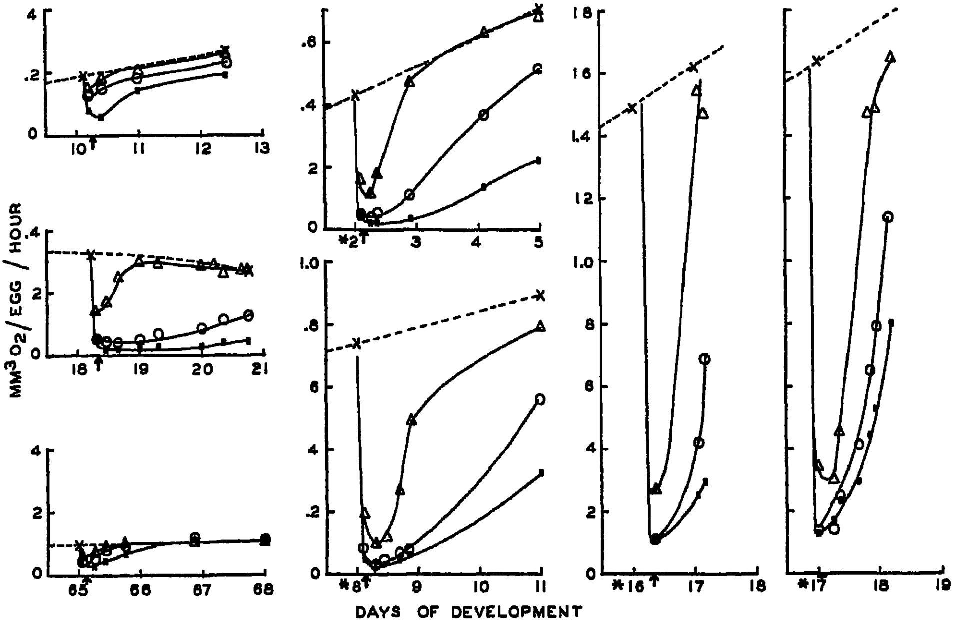


FIG. 3.—Exposure to and recovery from cyanide at different stages of development. Ordinates represent oxygen consumption in MM³ per egg per hour. Abscissae show the days of development. First numeral on abscissae shows age of eggs when cyanide was applied. Days marked with (*) indicate post-diapause age. × = control; △ = 0.0005M KCN; ○ = 0.0025M KCN; ■ = 0.01M KCN. Cyanide removed at arrow after 3-hour exposure.

Eighteen-day eggs are not only more depressed than those at the 10-day stage, but their recovery from exposure to cyanide is less rapid (Fig. 3). The former group treated for 3 hours at 25° C. with 0.01M KCN have recovered to 15 per cent of the original level of respiration at the end of 48 hours; the latter group have completely recovered by that time.

As one would expect, stronger concentrations of KCN depress respiration more than do weak ones. For the eighteenth day of prediapause development (Fig. 4*A*) the average residual respiration for 2 hours of a 3-hour exposure to concentrations of 0.01M–0.0005M KCN varies in a regular manner from 8.6 to 51.3 per cent of the control value.

---

[3] Whether there is a significant change in response after the formation of the chitinous cuticle on the sixth day (Slifer, 1937) is being investigated.

DIAPAUSE PERIOD

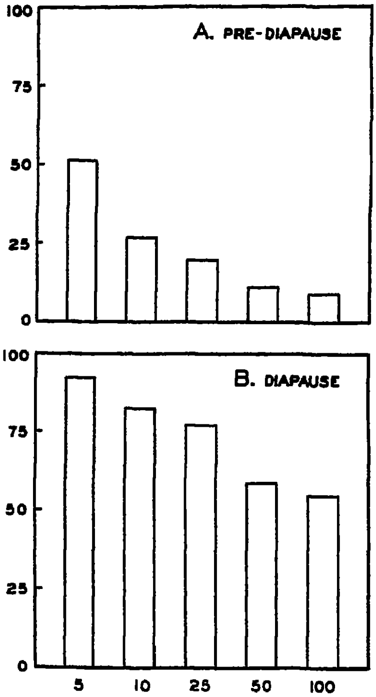


FIG. 4.—Effects of various concentrations of KCN on prediapause and diapause eggs. Exposures were for three hours at 25° C. with respiration measured over 2 hours of this period. Ordinates represent percentage residual respiration, abscissae show concentration of KCN × 10^{-4}. Each column includes: in (*A*) average of 5 lots of 18-day prediapause eggs; in (*B*) 6 lots of 70–120-day diapause eggs.

As pointed out in the preceding section the sensitivity to cyanide is markedly lessened when the egg enters the diapause or block period and the normal respiration decreases to the diapause level. This is shown graphically in Figure 2. A comparison of *A* and *B* in Figure 4 shows that 0.01M KCN does not have as great a depressing effect on diapause eggs as 0.0005M KCN has on 18-day prediapause ones, although the former solution is twenty times as concentrated as the latter and the two stages are very similar morphologically.

The effect of concentration on the diapause egg is graphically seen in Figure 4*B*. Residual respiration levels for concentrations of KCN varying from 0.01M to 0.0005M range from 54.7 per cent to 92.1 per cent of the control value. Diapause eggs behave similarly to those at the 10-day prediapause stage by recovering completely from a 3-hour exposure to 0.01M KCN within 48 hours (Fig. 3).

Responses of different lots of diapause eggs to KCN show considerable variation. From Figure 2 it may be seen that the residual respiration of eggs exposed to 0.001M KCN varies from 28 to 124 per cent of the control value. Although eggs in diapause are all at the same morphological stage of development, their sensitivity to cyanide seems to vary with the normal level of respiration in a manner similar to that of prediapause eggs (Figs. 2 and 5). Those lots of eggs which respire at low levels are very resistant to the action of 0.001M KCN, while the ones with comparatively high respiratory rates may be depressed more than 80 per cent. The greatest sensitivity to cyanide during the period of developmental block occurs between the twentieth and twenty-fifth days. This may be due to the fact that the level of respiration during the onset of diapause is higher than that characteristic of the diapause period in general. The indications are that cyanide sensitivity during both the diapause and the prediapause periods may vary directly with the respiratory rate. The curves in Figure 5 should not be considered as being in any way absolute boundaries between a cyanide-sensitive and a cyanide-insensitive portion of the respiration of the egg, as stronger concentrations of KCN produce further depression of the respiration in a still readily reversible fashion.

This increased cyanide sensitivity of eggs with a high respiratory rate as compared with those with a low rate might indicate that the potency of the diapause block is not as great in the former type of eggs as in the latter (Bodine and Boell, 1937). The increased respiration seems not to be associated with any visible morphological changes of the embryo, but may represent some type of physicochemical change similar to that which Runnström (1930) has suggested occurs in the sea urchin egg during transition from the unfertilized to the fertilized state.

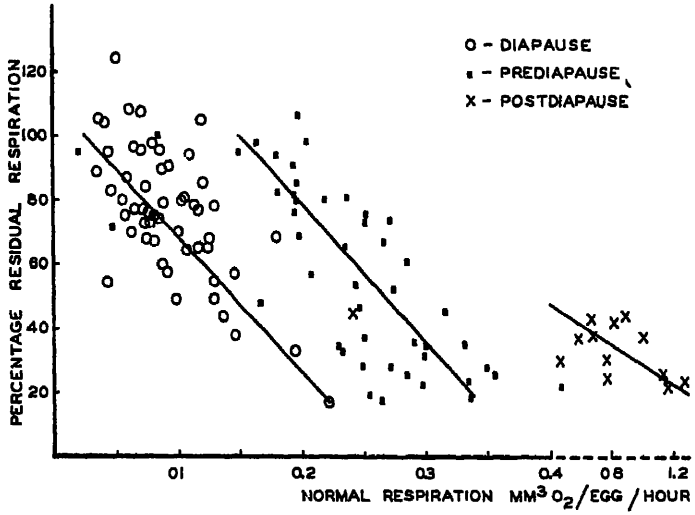


FIG. 5.—Curves showing variation in sensitivity to cyanide with variation in magnitude of respiratory rate. Ordinate represents percentage residual respiration; abscissa, respiratory rate. All eggs exposed for 3 hours at 25° C. to 0.001M KCN.

The similarity of response of 18-day prediapause and postdiapause eggs, and 10-day prediapause and diapause eggs (Figs. 2, 3, and 6) might suggest that there are two types of respiratory mechanisms active during embryonic development, one of which accounts for most of the respiration of the former group, the other for that of the latter. The stages between the tenth and eighteenth prediapause days and those diapause lots which have a higher respiration and are more sentitive to KCN than the normal may represent a combination of these two mechanisms. An investigation of this phase of the problem is being carried out at the present time.

POSTDIAPAUSE PERIOD

After the diapause block has been destroyed by exposure to low temperature and the eggs are again placed at 25° C., development is resumed and the respiratory rate rises rapidly. This is indicated graphically by the lower curve in Figure 2. The course of this curve representing normal egg respiration confirms the work of Bodine (1929) and Boell (1935).

It has been previously shown by Bodine (1934) that a marked susceptibility to KCN follows the termination of the diapause period. Confirmation of this is seen in the effect of 0.001M KCN on eggs treated within 24 hours of removal from the low temperature. Eggs so treated show a greater depression of respiration than any of those diapause eggs exposed to cyanide after the twenty-fifth day from laying (Fig. 2).

From the results of experiments graphically represented in Figures 2 and 6 it may be concluded that no significant change in sensitivity takes place during the postdiapause period. The level of residual respiration as percentage of the control value in the various concentrations of cyanide employed is about the same throughout the period as at the eighteenth prediapause day.

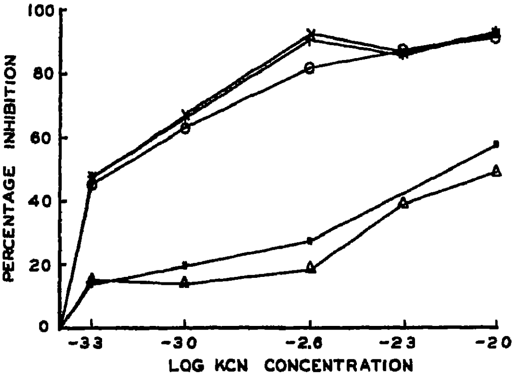


FIG. 6.—Effect of concentration of KCN. Points represent averages of experiments run with many different lots of eggs. All exposures were for three hours at 25° C. ■ = 10-day prediapause eggs; O = 18-day prediapause; Δ = diapause; + = 0-9-day postdiapause age; X = 9-18 day postdiapause.

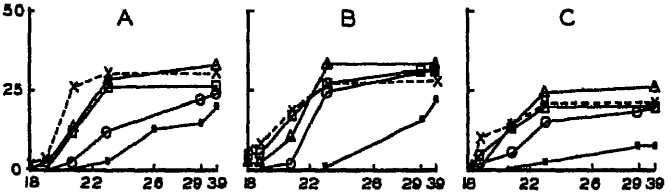


FIG. 7.—The effect of cyanide on time of hatching of eggs treated on zero postdiapause day (*A*), seventh day (*B*), and seventeenth day (*C*). X = control; Δ = 0.0005M KCN □ = 0.001M KCN; O = 0.005M KCN, ■ = 0.01M KCN. All exposures for 3 hours at 25° C. Ordinates represent number hatching; abscissae, postdiapause age.

Recovery of respiration after exposure of the eggs to cyanide proceeds more rapidly during the latter part of the postdiapause period than at the start (Fig. 3), but the delay in hatching caused by exposure is similar for eggs treated on the zero day of postdiapause development and those treated on the sixteenth day (Fig. 7). Those treated with 0.01M KCN hatch about a week later than the controls. The number of embryos which hatch is apparently not a good indication of the effect of cyanide on development; the embryos of those eggs treated after the middle of the postdiapause period, although delayed, progress in most cases to the morphological state typical of the eighteenth postdiapause day, but they seem to be unable then to escape from their shells. The fact that as many as 40 per cent of the postdiapause eggs treated for 3 hours at 25° C. with 0.01M KCN do hatch, and are able to live after hatching, shows that this concentration is at least partially reversible under these conditions.

SUMMARY

1. A study has been made of the effect of cyanide on the respiration of the egg of the grasshopper, *M. differentialis*, throughout its embryonic development, using the technique developed by Krebs and van Heyningen.

2. Methods of exposure to cyanide and their suitability for physiological uses are discussed.

3. An increasing sensitivity of eggs to cyanide is noted from the time of laying until the onset of diapause. Sensitivity to 0.001M KCN then decreases rapidly to a diapause level about 55 per cent less than the prediapause maximum.

4. During both the prediapause and the diapause periods the sensitivity seems to vary directly with the normal respiratory level of the eggs.

5. After diapause has been terminated by exposure to low temperature the sensitivity rises to about the same level as the prediapause maximum, and remains constant at this level.

6. Concentrations of KCN varying from 0.0005M to 0.01M show a progressively powerful action in depressing respiration. At the eighteenth prediapause day the inhibition produced by this range of KCN concentrations varies from 45 to 90 per cent of the control value. For the postdiapause period the range is from 47 to 92 per cent.

7. Ten-day prediapause and diapause eggs behave similarly toward cyanide, the inhibition varying from 14 to 57 per cent over the concentration range 0.0005—0.01M.

Curves showing recovery after exposure to KCN are given for prediapause, diapause, and postdiapause eggs.

9. A 3-hour exposure to KCN delays the time of hatching from a day to a week depending upon the concentration employed and regardless of the postdiapause age at the time of the application. This is true even if the exposure is made on the day immediately preceding that on which hatching normally occurs.

10. Three-hour exposures to 0.01M and 0.005M KCN result in a lowered percentage hatch, but the fact that 40 per cent or more of the control value of eggs hatch when treated with the former concentration shows that for this duration of exposure the dose is at least partially reversible.

LITERATURE CITED

BODINE, J. H. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zool., 2:459.

———. 1932. Hibernation and diapause in certain Orthoptera. II. Response to temperature during hibernation and diapause. Physiol. Zoöl., 5:538.

1934. The effect of cyanide on the oxygen consumption of normal and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., 4:397.

BODINE, J. H., and BOELL, E. J. 1934. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol, 5:97.

———. 1937. The action of certain stimulating and inhibiting substances on the respiration of active and blocked eggs and isolated embryos. Physiol. Zoöl., 10:245.

Boell, E. J. 1935. Respiratory quotients during embryonic development (Orthoptera). Jour. Cell. and Comp. Physiol., 6:369.

Buchanan, J. W. 1926. Depression of oxidative metabolism and recovery from dilute KCN. Jour. Exper. Zool., 44:285.

Dixon, M. 1929. Oxidative mechanisms in animal tissues Biol. Reviews, 4:352.

Dixon, M., and Elliott, K. A. C. 1929. The effect of cyanide on the respiration of animal tissues. Biochem Jour., 23:812.

Heyningen, W. E. van. 1935 The inhibition of respiration by cyanide. Biochem. Jour , 29:2036.

Krebs, H. A. 1935. Metabolism of amino acids. III. Deamination of amino acids Biochem. Jour , 29:1620.

Meldrum, N. U. 1934. Cellular respiration. London: Methuen.

Runnstrom, J. 1930. Atmungsmechanismus und Entwicklungserregung bei dem Seeigelei Protoplasma, 10:106.

Schmitt, F. O., and Schmitt, O. H. A. 1931 The nature of the nerve impulse. II. The effect of cyanide upon medullated nerves. Amer. Jour. Physiol., 97:302.

Slifer, E. H. 1932. Insect development. IV. External morphology of grasshopper embryos of known age and with a known temperature history. Jour. Morph., 53:1.

———. 1937. The origin and fate of the membranes surrounding the grasshopper egg; together with some experiments on the source of the hatching enzyme. Quart. Jour. Mic. Soc., 79:493.

Smith, R. G. 1929. A method for the quantitative determination of cyanide in small amounts. Jour. Amer. Chem Soc., 51:1171 (Part I).

Warburg, O. 1928. Über die katalitischen Wirkungen der lebendigen Substanz Berlin: Julius Springer.

COMPARISON OF QUANTITATIVE PRECIPITIN TECHNIQUES AS INFLUENCED BY INJECTION PROCEDURES

(Seven figures)

HAROLD R. WOLFE AND JOSEPH G. BAIER, JR.
Department of Zoölogy, University of Wisconsin

THE specificity of the precipitin reaction has been found to be altered by continued injections of antigen. Magnus demonstrated this in 1908 using plant tissue extracts. Lake, Osborne, and Wells in 1914 showed that, when purified vegetable proteins were used as antigens, the specificity of the resulting immunological reactions, as measured by precipitin, anaphylactic, or complement fixation tests, was altered by continued injections. Satoh (1933) distinguished between *präzipitingehalt* and *präzipitintiter* and found the number of injections affected the former considerably. Antisera produced by the larger number of injections were less specific and were not so easily affected by higher dilutions as were the antisera resulting from fewer injections. Hooker and Boyd (1934) using crystalline albumen of duck and hen eggs found that earlier bleedings exhibit more specificity than later bleedings.

Quantitative studies of the effects of injection procedures on the serum precipitin reaction have been reported by Wolfe (1935, 1936). In these experiments he used rabbits as antibody builders and the serum proteins of various mammalian bloods as antigens. When small quantities (2–3 cc.) of undiluted serum proteins were injected in three doses into rabbits, the resulting antisera were more specific than those produced by injections of 10 cc. or more in five to ten injections. Antisera produced by the former method usually yielded higher titered reactions but seldom gave "extra-group" response. Reinjections of the animals with 1.5 cc. of undiluted homologous antigen at intervals of a month or more resulted in the production of aspecific precipitins, but reinjections with minute quantities restored the antibody content of the blood without affecting the quality of the precipitins. These data were believed to be of especial importance to the serologist who is interested in studies of animal or plant relationships. Greater uniformity of results could be expected when injection procedures were controlled and the value of the precipitin test in studies on phylogeny would be enhanced.

If the antisera were produced by injections of the smaller quantity of antigen or by reinjections of minute quantities, the ring-test titers were found to be much higher than the flocculation titers after 1 hour of incubation at 37.5° C. ± 1. It was also noted that the amount of the precipitate of an antigen-antibody mixture, as determined macroscopically, varied with the amount and number of injections. Increased quantities of antigen injected into a rabbit and additional series of reinjections of undiluted antigen resulted in greater amounts of precipitate in the tests performed in vitro. There was no noticeable correlation between "ring-test" titer and precipitate formation. The interfacial titer reached a maximum after one or two injection series, but the precipitating power seemed to increase with each reinjection of undiluted antigen.

There are various quantitative methods for studying the precipitin reaction. The more accurate ones employ measurements of the precipitate by (1) direct volumetric determination, (2) dry-weight measurement, and (3) nitrogen analysis. The greater reliability of one technique over the other has not been established to the satisfaction of all workers. All these techniques are different expressions of the amount of precipitate formed, and this, it is assumed, is an index of the quantity of antibody present in an antiserum. It is known that the precipitate consists of antigen and antibody. Therefore, in an antigen-antibody system, at points of optimal proportions where the supernatant fluid contains practically no antigen or antibody, if the antigen is constant and the amount of precipitate varies, it is an indication of the variance in the amount of antibody.

Theoretical considerations of the precipitin system have been made by Heidelberger and his co-workers. They have employed various simple antigens and have concluded that the reaction follows the law of mass action. Heidelberger, Kendall, and Soo Hoo (1933) reported the effects of successive courses of injections on the precipitin content of the rabbit's blood. They found two types of response. In one type of response the rabbit's blood reached a relatively low but maximum content with one course of injection, and a second type of response occurred in which the precipitin content increased with successive courses to a maximum. Their data almost entirely substantiated their first point. Taylor, Adair, and Adair (1934) and Heidelberger and Kendall (1935) reported effects of more than one course of injections. The latter authors concluded from their results and the results of Taylor, Adair, and Adair that "the sera exhibited progressive changes. . . . . These changes are believed to consist in the formation of more and more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule." These results can be readily understood and are not unexpected, for the rabbits were injected for long periods of time with a comparatively large amount (total) of antigen. The greatest possible antibody response should be expected with such treatment. The studies did not include the earlier stages of precipitin formation in the rabbit.

Baier (1933) has been able to show graphically the relations of antigen and antibody in the precipitin reaction and in what respect various chemical and physical agents may modify their interaction. The technique employed in this study was reported by Boyden and Baier (1929) and is concerned with volumetrically measuring the amount of precipitate formed in the reaction between known amounts of an antigen with its specific antiserum by means of Van Allen thrombocytocrits. They have shown their quantitative volumetric technique to be fairly simple, rapid, and statistically reliable.

The purposes of the research work described in this report are to show, by the use of a volumetric quantitative technique of known accuracy, (1) the effects of injection procedures on the precipitating power of antiserum from the initial appearance of antibody through successive courses of injections, (2) a comparison of the volumetric precipitate formation and ring-test titer, and (3) a correlation of these results with previous work.

MATERIALS AND METHODS

Sterile buffered saline solutions of egg albumen, beef oxyhemoglobin, and the albumen and pseudoglobulin fractions of pig and dog sera were used as antigens and injected into healthy rabbits. The initial series of injections consisted of three doses given on alternate days; the first dose consisting of 0.5 cc. and the total amount 3 cc. If the titer ("ring" test) was then sufficiently high, the animals were bled 12 days after the last injection. Then, at intervals of a month or longer, the animals were given repeated series of reinjections, each series consisting of 1.5 cc. of undiluted homologous antigen administered in two doses on alternate days. Ten days after the last series of reinjections, the animals were bled. In several cases the animals were also bled just previous to each reinjection series.

The protein content of the solutions used was determined by the modified Macro-Kjeldahl method and was based on the total nitrogen content. The antigens varied from 0.94 to 5.11 gm. protein per 100 cc. of solution, and this was considered the undiluted antigen. Two per cent standard solutions (1:50 dilution of the undiluted antigen with buffered saline) were made, and for ring-test titers and volumetric determinations these solutions were used in serial dilutions. The crystalline egg albumen was prepared according to the method of Hopkins and Pinkus (1898), and the oxyhemoglobin after the method of Marshall and Welker (1913). The individual proteins of the blood serum were fractionated by the procedures described by Hektoen and Cole (1927).

Each rabbit was given a definite number, and the initial antiserum obtained was numbered similarly. The letter "A" was added to this number to indicate that the blood was taken from the rabbit after a rest period of 30 or more days and the number "1" added if the animal had been reinjected 10 days previously. Thus antiserum ♂152-A1 was from a male rabbit bled for the third time and taken 10 days after the second injection series. The sera were stored in ampules after sterile filtration and kept at lowered temperatures.

Reactions were determined only for homologous antigens. The term "titer" when used in this paper refers to the ring-test titer, while the term "precipitating power of an antiserum" refers to the production of a volumetric measurable amount of precipitate in an antigen-antibody mixture. To understand the volumetric data as presented in this paper, suppose we interpret the curves from one set of volumetric determinations, such as in Figure 3. Beginning at the junction of the abscissa and the ordinate and progressing toward the direction of greater antigen dilution (lesser antigen concentration) we can see that the volume of precipitate rapidly reaches a maximum and then gradually decreases. The part of the curve to the left of the maximum represents the "zone of inhibition," where the antigen, being in excess, has caused a re-solution of the precipitate. The part of the curve to the right represents the volume of precipitate obtained when the antigen is relatively deficient. The peak of the curve is relatively at a concentration of antigen and antibody where neither one is in excess. Therefore, a shift in this peak to the left, where the antigen concentration becomes greater, shows an increase in "precipitating antibody." Also, an increase in the amount of precipitate produced shows an increase in "precipitating antibody," so that either one, or both, of these factors may be employed in describing the potency of an antiserum in its precipitating power (Baier, 1933, Fig. 6).

RESULTS

Table 1 records data on antisera produced by seven rabbits, and exemplifies the method of numbering of the antisera. Five other rabbits giving similar results are not listed. The dates of injections and of bleedings should be correlated with the interfacial test titer and with the volumetric method of measuring the precipitate. While antisera of very high titer did not necessarily yield a measurable precipitate, precipitating sera always were of high titer. One injection series often resulted in a maximum titer, but at times two series were necessary (especially against hemoglobin) for its production. Further reinjections did not raise the titer but usually resulted in an increased amount of precipitate as determined by measurements with the thrombocytocrits. It may also be noted that, if a rabbit is not reinjected for a long period of time (a month or more), its serum will show a loss of "precipitating power," but the "titer" will not have decreased as greatly.

TABLE 1

EXPERIMENTAL DATA—ANTIGENS USED AND "PROPERTIES" OF ANTISERA

Antiserum Number	Antigen Injected and Protein Content	Date of Injection*	Date of Bleeding	Titer	Measurable Precipitate Formation	Refer to Fig. No.
♂155	Beef oxyhemoglobin 5.11 gm. per 100 cc.	1-14-35	1-30-35	0	−	....
-I		2-23-35	3-6-35	12,800	−	....
-II		4-1-35	4-12-35	25,600	+	1
-IIA		....	7-1-35	6,400	−	....
-IIAI		7-11-35†	7-23-35	12,800	−	....
-IIAIA		....	10-9-35	6,400	−	....
-IIAIAI		11-13-35	11-24-35	25,600	+	1
♂170		3-11-35	3-25-35	400	−	....
-I		4-15-35	4-27-35	25,600	−	....
-II		5-20-35	6-1-35	51,200	−	....
-III		8-21-35	9-2-35	51,200	+	2
-IIII		11-25-35	12-7-35	51,200	+	2
-IIIIA		....	2-17-36	12,800	−	....
-IIIIAI		2-29-36	3-12-36	51,200	+	2
♂152	Crystalline egg albumen, 4.05 gm. per. 100 cc.	1-14-35	1-30-35	25,600	−	....
-I		2-23-35	3-6-35	51,200	+	3
-II		4-1-35	4-12-35	51,200	+	3
-IIA		....	8-1-35	25,600	−	....
-IIAI		7-11-35†	7-23-35	25,600	−	....
♀167		3-4-35	3-20-35	51,200	−	....
-I		4-16-35	4-28-35	51,200	+	4
-II		5-20-35	6-1-35	51,200	+	4
♀163	Albumen fraction of dog serum, 3.55 gm. per 100 cc.	3-4-35	3-20-35	25,600	+	5*a*
-I		4-15-35	4-27-35	25,600	+	5*a*
-II		5-22-35	6-3-35	25,600	+	5*a*
-IIA		....	8-22-35	25,600	−	....
-IIAI		9-4-35	9-16-35	25,600	+	5*b*
-IIAII		12-2-35	12-14-35	25,600	+	5*b*
♀173		4-1-35	4-15-35	800	−	....
-I		5-6-35	5-18-35	25,600	−	....
-II		6-17-35	6-29-35	51,200	+	6
-III		7-25-35	8-6-35	51,200	+	6
-IIIA		....	10-21-35	12,800	−	....
♂156	Pseudoglobulin fraction of dog serum 0.94 gm. per 100 cc.	3-11-35	3-25-35	1,600	−	....
-I		4-16-35	4-28-35	6,400	+	7
-II		5-20-35	6-1-35	25,600	+	7
-IIA		....	8-24-35	800	−	....

* First injection of series.

† 2 per cent solution injected instead of undiluted antigen.

Antibeef oxyhemoglobin sera from two rabbits yielded very similar results. It was necessary to inject rabbits ♂155 and ♂170 with two series of antigens before they responded with high-titered antisera. Further reinjections with undiluted antigen did not increase this value (within the range of error of the test which is one tube). A measurable precipitate was obtained after three injections into rabbit ♂155 and after four series in rabbit ♂170. Figure 1 shows that further treatments with undiluted antigen increased the quantity of antibody in the antigen-antibody mixture as indicated by a shift of the curve to the left. Figure 2 demonstrates the increased antibody content of series ♂170 resulting from successive courses of injections, for there is a larger quantity of precipitate formed as well as a shift of the peak of the curve to the left. Antiserum ♂155-11A1 (Table 1) was produced by reinjections with a 2 per cent solution, but it did not have any "precipitating power." The results obtained with antisera ♂155-11A, ♂155-11A1A, and ♂170-1111A (Table 1) illustrate the effects that a long period of rest, occurring after injections, have on "titer" and "precipitating power." The type of curve resulting when hemoglobin was used as antigen was different from the curves obtained by the use of other antigens. Analyses of these differences have not been made.

Figures 3 and 4 are the precipitate curves for two series of antisera against crystalline egg albumen. The initial series of injections in each rabbit produced high-titered antisera without producing measurable amounts of precipitate. Reinjections of the two rabbits about a month later with 1.5 cc. of undiluted antigen resulted in antisera that yielded much precipitate, but whose titers did not change outside the limits of error of the test.

Serum ♂152-2 gave a much greater precipitate than did ♂152-1. In order to obtain accurate measurements with ♂152-2, it was necessary to dilute one volume of it with one volume of buffered saline (resulting dilution 1:2), for without dilution the thrombocytocrits that were used at the time were not large enough to measure the precipitate obtained from a mixture of antibody and antigen at the point of highest precipitate formation. The effect of antiserum dilution as seen in the comparison of graphs ♀163 undiluted and ♀163 diluted 1:2 (Fig. 5*a*) is to lower proportionately the amount of precipitate obtained volumetrically and to shift the peak to the right without otherwise altering the characteristics of the curve. The peak of the curve for ♂152-2 is also much to the left of the peak for ♂152-1, even though the former was diluted 1:2. This is similar to the results shown in Figures 1 and 2. When the rabbit was bled about 3 months after the ♂152-2 bleeding, its blood was still of high titer but lacked precipitating power, which was not restored with reinjections of 1.5 cc. of a 2 per cent antigen solution.

The volume of precipitate of ♀167-2 and its homologous antigen was considerably greater than that of ♀167-1 when used undiluted. The former yielded measurable quantities of precipitate when diluted with three parts (1:4 dilution) of buffered saline, and without dilution the volume of precipitate obtained was so great as to be immeasurable.

Two rabbits were injected with the albumen fraction of dog serum. The initial set of injections into rabbit ♀163 resulted in a titer of 1:25,600, and four series of reinjections with undiluted antigen had no effect on this value. Serum ♀163 was the only one that yielded a precipitate (Figs. 5*a* and 5*b*) following one series of injections. The first and second reinjection series increased the amount so greatly that the antisera had to be diluted in order to allow for volumetric determinations. On the other hand, the third and fourth series of reinjections did not increase the quantity of precipitate. Usually the measurements of precipitate were made within 2 or 3 months after the antiserum was secured, but in these cases the determinations were made after 18 months had elapsed. At the present time it is not known whether or not this result indicates that the precipitating

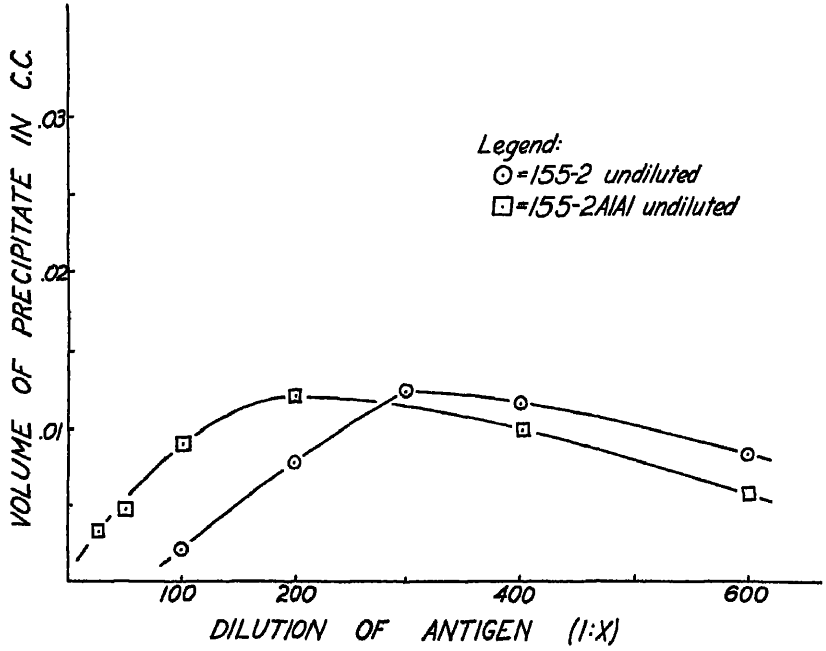


FIG. 1.—Antiserum of rabbit ♂155 against antigen BH-100

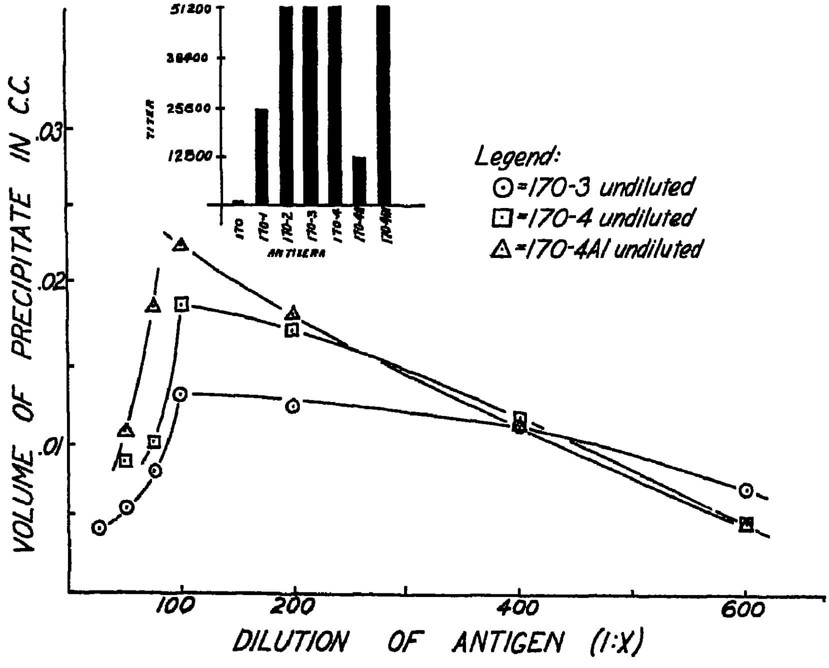


FIG. 2.—Antiserum of rabbit ♂170 against antigen BH-100

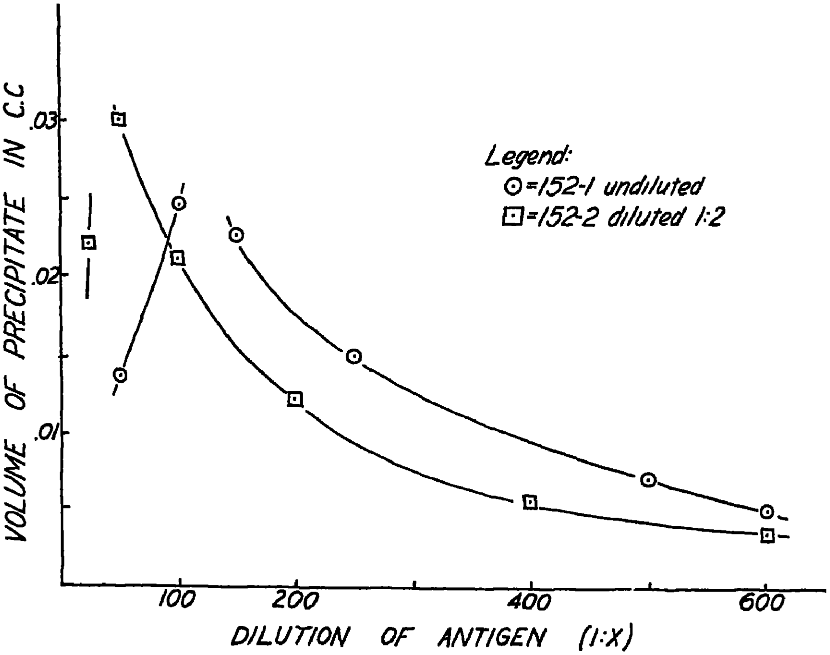


FIG. 3.—Antiserum of rabbit ♂152 against antigen CE-21

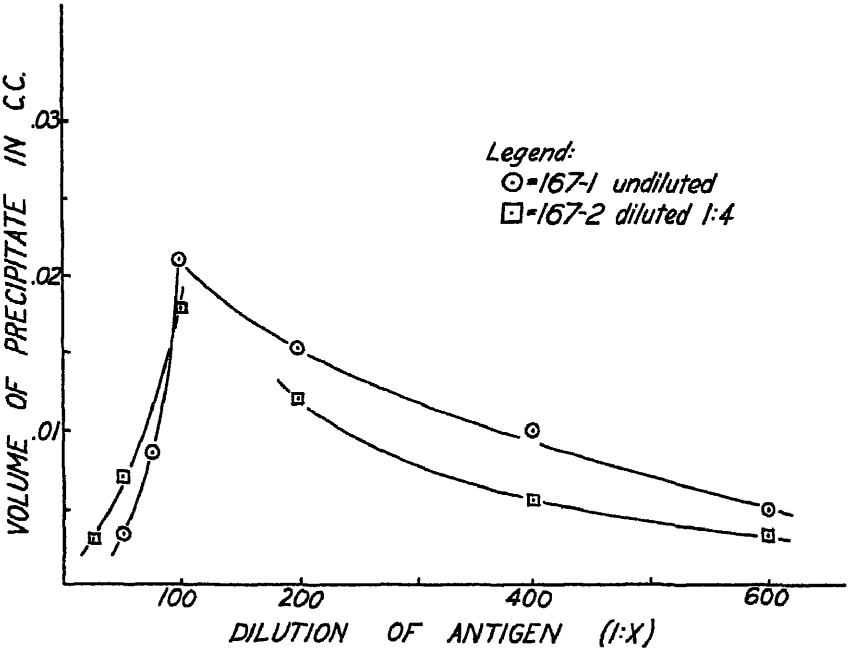


FIG. 4.—Antiserum of rabbit ♀ 167 against antigen CE-21

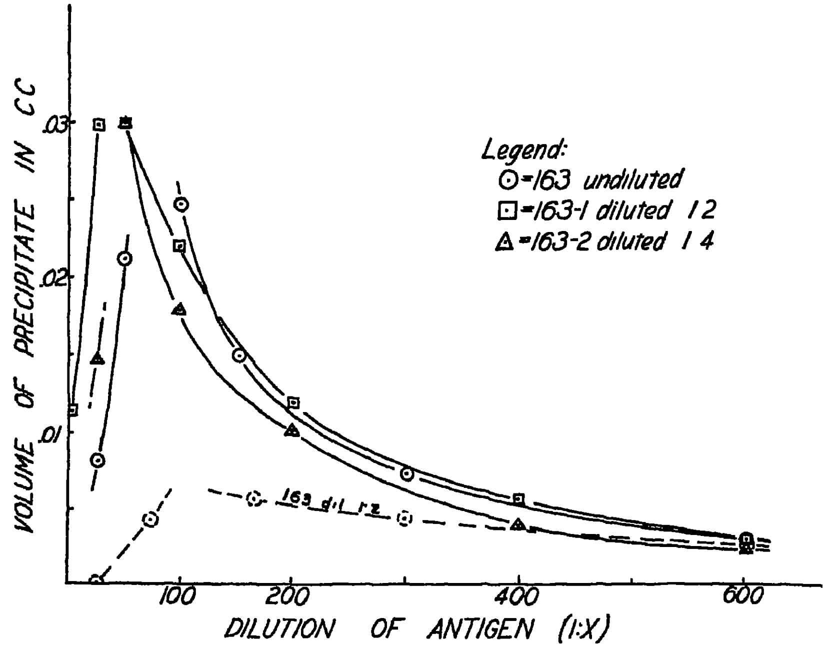


FIG. 5a.—Antiserum of rabbit ♀ 163 against antigen DA-1

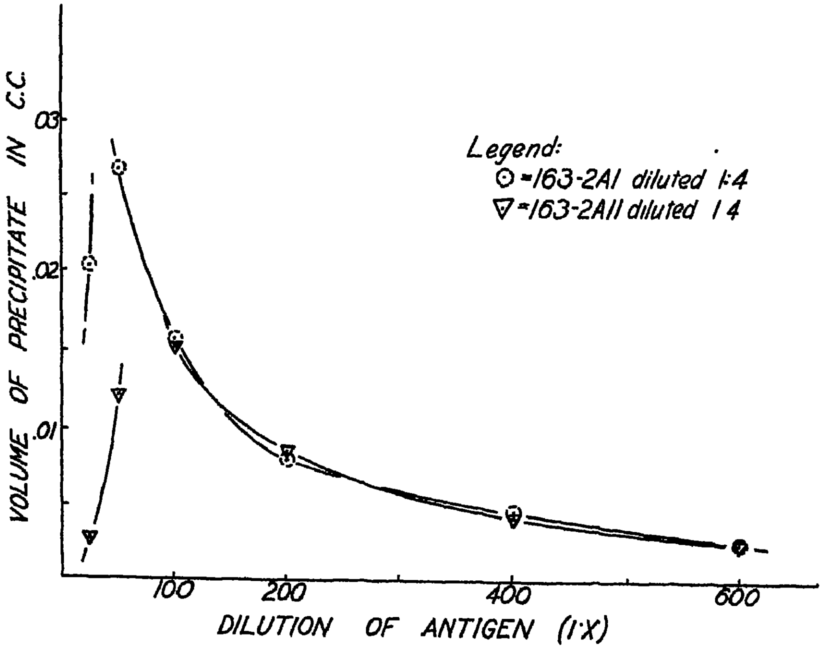


FIG. 5b.—Antiserum of rabbit ♀ 163 against antigen DA-1

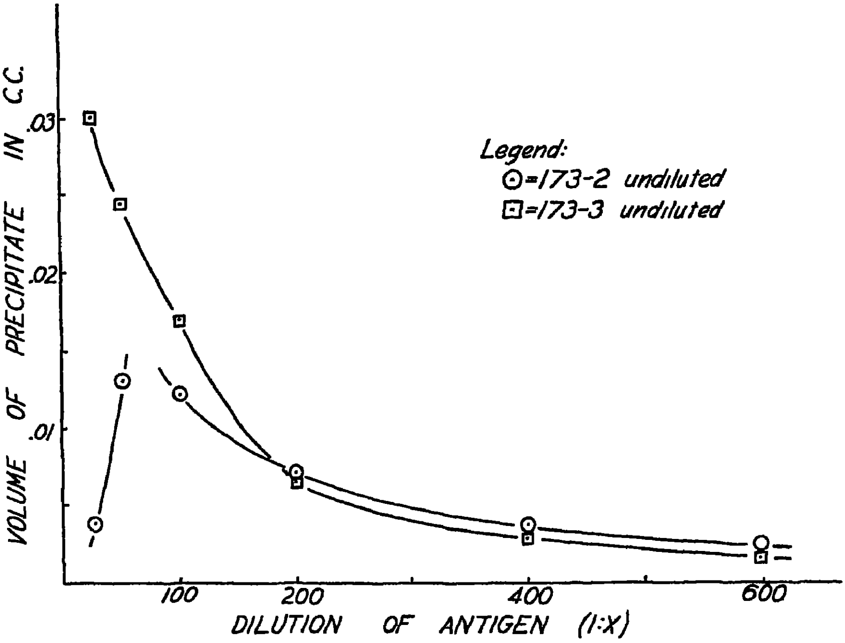


Fig. 6.—Antiserum of rabbit ♀173 against antigen DA-1

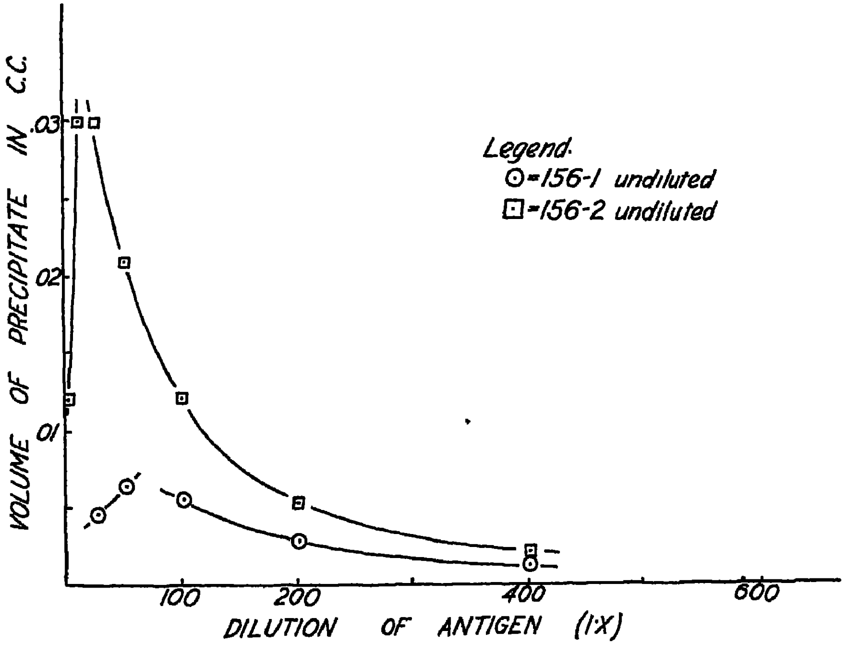


Fig. 7.—Antiserum of rabbit ♂156 against antigen DP-1

power of a serum reaches a maximum and cannot be raised above this point, or whether other factors, such as age of the antiserum, may affect the results. These factors are being studied in further experiments.

Figure 6 records the precipitating curves for two antisera against the albumen fraction of dog serum, and Figure 7 represents curves for antisera against the pseudoglobulin fraction. Again, the effects of reinjection with undiluted antigen have been shown to cause an increase in the precipitating power of an antiserum when reacted against its homologous antigen, while without reinjection, for a month or longer, this power of the rabbit's serum to form a precipitate decreases (Table 1).

DISCUSSION

The experiments have been carried out in two steps. One of the authors injected the animals and made the ring-test titers, while the other, not knowing the procedures or results of this, made the volumetric determinations. The tabulation and correlation of the results showed a noteworthy consistency of the data. Certain facts that have not been fully dealt with by other workers were demonstrated in regard to the effect of injection methods on the properties of precipitins.

It can be observed from a study of any one of the figures that the first precipitating antiserum obtained from a rabbit has the highest peak of the curve, showing maximum precipitate formation at a point where the antigen dilution is of a relatively low concentration, while the same rabbit serum obtained after a second and third series of injections shows its point of maximum precipitate formation at a lower dilution (higher concentration of antigen). In attempting to explain this, let us recall that the "zone of inhibition" is that portion of the curve where the antigen is in excess, and which therefore causes re-solution of the precipitate. As a result, the antisera of the second and succeeding injection series must contain a greater quantity of precipitating antibody, for in many graphs the "zone of inhibition," in spite of using diluted antisera, continues to be moved toward the left of the graph. That there is no exact correlation between the increase in precipitating power of an antiserum and its "titer" is readily shown from this analysis of the curves. This is suggestive that there are two kinds of antibodies produced —the "titer"-producing antibody and the "precipitate"-forming antibody. A study of this is now being made.

It is an interesting fact that, with the methods and materials used, the ring-test titers early reached a maximum and could not be raised while the volume of precipitate increased with injections of undiluted antigen. Is it possible that the titer could not be raised because the amount of protein in the higher dilutions was infinitesimally small? The facts clearly demonstrate the differences between the "titer" of an antiserum and its ability to precipitate in the presence of varying amounts of an antigen. A very similar variation is reported by Wolfe (1935, 1936), where an antiserum may at one time have a high titer and be very specific toward its antigen but on further injection of the same rabbit producing this antiserum still have the same high titer and yet be very aspecific.

Apparently these methods of study are showing us more conclusively that antisera to be comparable must be produced following definite procedures depending upon the "type" of antiserum desired. To the zoölogist who is studying animal relationships the ring-test method seems most applicable. If such factors as injection procedures and protein content of antigen, which was emphasized by Boyden (1926), are taken into consideration, the value of the precipitin test in such studies would be greatly increased.

The medicolegal worker would also benefit were he to consider these factors. Antisera that yield measurable amounts of precipitate give valuable data in interpreting the mechanism of the reaction, but whether this type of antiserum can show, as accurately as the "ring" test, phylogenetic relationships has not been shown. It would be of interest and significance to know how the variance in quantity of antibody in antisera would affect the cross-reactions when the antisera give measurable precipitates.

Six monkeys, the weights of which were approximately the same as those of the rabbits, were injected and reinjected with undiluted horse serum. The quantities injected were the same as those used in rabbits, but the protein content of the serum was slightly higher. The monkeys received from five to eight series of injections. The responses were very different from those obtained in the rabbits. The titers were weak, with a maximum of 1:12,800, but more usually of 1:3,200. There was very little precipitate formation, and the antiserum was very specific in the few cross-tests that were made. A comparison of the monkey and rabbit, of course, demonstrates that, when horse-serum proteins are used and injection methods are similar, the formation of precipitins is very different in the two species. This is a species difference as far as we can determine, unless factors that we haven't anticipated have been overlooked. What is the cause of this difference in response? We find no answer but use the oft-repeated term "species individuality."

SUMMARY

1. Rabbits have been injected with mammalian blood proteins and crystalline egg albumen.

2. High-titered antisera were produced by one or two series of injections of undiluted antigen, and this titer could not be raised by further injections.

3. Continued reinjections, as far as the experiments went, resulted in increased precipitate formation and a shift of the curve toward the region of greater antigen concentration showing an increase in the antibody causing precipitation.

4. Two injection series were usually necessary to produce antisera that had precipitating power; using hemoglobin solutions, three and four injection series were needed.

5. The precipitating curves resulting with hemoglobin as antigen differed from the type of curves with the other antigens.

6. Indications are that at least two types of antibodies are present: (1) a "titer"-producing antibody and (2) a "precipitate"-forming antibody.

7. The zoölogical significance of the data is emphasized.

LITERATURE CITED

Baier, J. G., Jr. 1933. Quantitative studies on precipitins. Physiol. Zoöl., 6:91.

Boyden, A. A. 1926. The precipitin reaction in the study of animal relationships. Biol. Bull., 50:73.

Boyden, A. A, and Baier, J G., Jr. 1929. A rapid quantitative precipitin technic. Jour. Immun, 17:29.

Heidelberger, Michael, and Kendall, Forrest E. 1935. A quantitative theory of the precipitin reaction, III: The reaction between crystalline egg albumin and its homologous antibody. Jour. Exper. Med., 62:697.

Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M. 1933. Quantitative studies on the precipitin reaction. *Ibid.*, 58:137.

Hektoen, L., and Cole, A. G. 1927. The preparation and precipitin reactions of egg albumin and blood proteins of the domestic fowl. Jour. Inf. Dis., 40:647.

Hooker, S. B., and Boyd, W. 1934. The existence of antigenic determinants of diverse specificity in a single protein, II: In 2 natural proteins: crystalline duck egg albumin and crystalline hen egg albumin. Jour. Immun., **26**:469.

Hopkins, F. G., and Pinkus, S. N. 1898. Observations on the crystallization of animal proteids. Jour. Physiol., **23**:130.

Lake, G. C., Osborne, T. B., and Wells, H. G. 1914. The immunological relationship of Hordein of barley and Gleadin of wheat as shown by the complement fixation, passive anaphylaxis, and precipitin reactions. Jour. Inf. Dis., **14**:364.

Marshall, J., and Welker, W.H. 1913. Precipitation of colloids by means of aluminum hydrate. Jour. Amer. Chem. Soc., **35**:820.

Satoh, T. 1933. Ueber Präzipitintiter und Präzipitingehalt. Zeitsch. f. Immunitätsforsch. u. exp. Therapie, **79**:117.

Taylor, G. L., Adair, G. S., and Adair, Muriel E. 1934. The precipitation reaction, optimal proportions, neutrality and maximal precipitation in mixtures of albumin and antiserum. Jour. of Hyg., **34**:118.

Wolfe, Harold R. 1935. The effect of injection methods on the species specificity of serum precipitins. Jour. Immun., **29**:1.

———. 1936. The specificity of precipitins for serum. *Ibid.*, **31**:103.

THE INFLUENCE OF THE FREQUENCY OF STIMULATION ON THE SLOW AND THE FAST CONTRACTION IN CRUSTACEAN MUSCLE

(Two figures)

C. A. G. WIERSMA AND A. VAN HARREVELD
William G. Kerckhoff Laboratories of the Biological Sciences,
California Institute of Technology, Pasadena, California

AS WAS shown by the authors (1936, 1938), most muscles of the extremities of decapod crustaceans have a double motor innervation, each muscle being innervated by two axons which have different functions. The thicker of the two fibers causes a fast, the thinner one a slow, contraction. In most cases both kinds of contraction need more than one nerve impulse before a mechanical response is obtained. The only exception found so far is the adductor muscle of the crayfish claw (van Harreveld and Wiersma, 1936, and Wiersma, 1937), in which a single impulse in the thick fiber elicits a strong twitch. Stimulating the thin fiber for the adductor muscle, a mechanical response can be obtained only after a number of impulses have reached the muscle. In *Blepharipoda occidentalis* a mechanical response was seen after stimulating the thick fiber with two induction shocks following each other with an interval of 2–10 σ (Wiersma and van Harreveld, 1938). Stimulation with two shocks at longer intervals does not elicit a mechanical response, but with repetitive stimuli a contraction is still obtained with intervals up to 50 σ. A single nerve impulse does elicit an action current of considerable size, however. It was found that in *Blepharipoda* the slow contraction was larger than the fast one up to frequencies of 50 per second. In the adductor of the walking leg of *Cancer anthonyi*, in which the fast mechanical contraction also needs at least two nerve impluses, the fast contraction is higher than the slow one only with frequencies above 100 per second. As these phenomena were considered of importance for the relation between the slow and the fast contraction, a number of experiments were carried out on this line to obtain further evidence.

EXPERIMENTS WITH *Blepharipoda*

The method used was the same as described in previous papers. The thick and the thin fiber were isolated and each placed on a pair of micromanipulated electrodes. By turning a double switch one or the other could be connected with a vacuum-tube stimulator, supplying short ($\frac{1}{2}$ σ) rectangular currents at various frequencies. The recording of the mechanical contraction was carried out by a light auxotonic lever to which a small mirror was fastened, reflecting a beam of light to the slit of the camera used for the recording of the electrogram. Since the distance between the mirror and the camera was 3 m., the recording was very sensitive and indicated even the slightest contraction. The muscle-action currents were led off with chloride-plated silver electrodes and recorded with a Matthews oscillograph.

Starting from a frequency of 50 per second, at which, as we have shown (1938), the mechanical contraction caused by either of the fibers is roughly the same in this animal, lowering the frequency gives an increased difference between the fast and the slow contraction, the fast becoming weaker and weaker in relation to the corresponding slow contraction. It was found, however, that as long as there is a contraction on stimulation of the thick fiber this occurs rather quickly, whereas slow contractions become apparent only after prolonged stimulation when the frequency is quite low.

At a certain frequency the fast contraction is no longer demonstrable whereas the same stimulation still gives a very distinct slow contraction. Since the action potential caused by a single impulse is quite large, as stated above, the action currents of the fast contraction are of course very distinct. The action currents obtained from the slow contraction at these frequencies are so poorly facilitated that they are very small and difficult to record. Thus the paradoxical phenomenon was found that a muscle giving relatively high action currents on stimulation of the thick fiber does not show any contraction, whereas on stimulation of the slow fiber much smaller action currents were elicited, causing, however, a very distinct contraction. We will refer to this phenomenon, which is illustrated in Figure 1 as the "paradox." It will be clear that this paradox can be obtained with any frequency between the minimum for the slow contraction and that of the fast contraction. In this animal the minimum for the slow contraction is about 10 per second, that of the fast 20 per second.

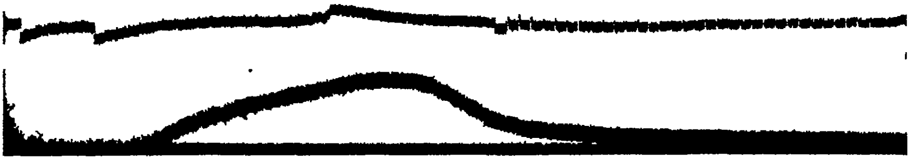


FIG. 1.—*Blepharipoda occidentalis*, adductor muscle, electro- (*a*) and mechanogram (*b*). Paradox with a stimulating frequency of 12 per sec. *A*, thin axon stimulated, action currents are very small, hardly detectable, contraction after a long latent period. *B*, stimulation of thick fiber, large action currents, no contraction, instead of which the relaxation of the slow contraction still continues. Notice the facilitation of the action currents. Time mark is $\frac{1}{5}$ of a sec.

It was mentioned previously (1938) that the action currents of the fast contraction show facilitation when stimulated with frequencies of more than 1 per second. This facilitation depends only on the frequency of the stimulus; the higher the frequency the larger the facilitation. At the minimum frequency causing a mechanical response no sudden change of the facilitation is observed.

Another point which we investigated in this animal in relation to the mechanism of slow and fast contraction was latent facilitation. We have repeatedly pointed out that the slow action currents obtained after a short period of rest after a previous contraction grow faster than without a previous stimulation, as does the contraction. We confirmed this for the slow contraction in this animal and found it also to be true for the fast action currents. Working with a frequency at which no contraction took place (13 per second), we found that when two rather short stimulations were given with an interval of 1.5 seconds the first action current had in both cases the same height but the growth of the action currents was quicker during the second stimulation (Table 1). With a time interval of 10 seconds this latent facilitation on a stimulation time of the same length had disappeared.

EXPERIMENTS WITH *Cambarus* AND *Astacus*

Under normal circumstances it is of course impossible to obtain a paradox in these animals, as the fast contraction needs only one nerve impulse. As was shown by Wiersma (1933), the mechanical contraction following a single nerve impulse can disappear when the stimulus is repeated a number of times with long intervals between successive stimuli, in which case the action current remains. The muscle then still contracts after two stimuli in close succession or during faradic stimulation. Wiersma found that the contraction disappeared in *Astacus fluviatilis* without any apparent change in the action current. We repeated these experiments both in *Cambarus* and in *Astacus trowbridgii*. In *Cambarus* the action currents diminish always, and sometimes even markedly, during the procedure, but in *A. trowbridgii* we were able to confirm his results as here there is often only a negligible reduction in their height. As was the case in *Blepharipoda*, there is no relation between the absence of the contraction and the reduction of the action current; if, for instance, the stimulation is continued after any trace of the contraction has disappeared, the action current still goes on diminishing at about the same rate as before (see Table 2).

TABLE 1

Blepharipoda occidentalis: STIMULATION OF THE THICK AXON OF THE ADDUCTOR WITH A FREQUENCY OF 13/SEC.

	HEIGHT OF SPIKE (IN MM.)					
	First Impulse	Second Impulse	Third Impulse	Fourth Impulse	Fifth Impulse	Ninth Impulse
First stimulation	12.2	13.1	14.0	14.5	14.9	16.0
Rest 1½ sec.						
Second stimulation	12.2	13.8	15.0	15.2	15.4	16.9
Rest 5 min.						
Third stimulation	12.2	13.3	14.2	14.5	14.9	15.9
Rest 10 sec.						
Fourth stimulation	12.3	13.0	14.0	14.5	15.0	16.0

In a preparation in which the mechanical contraction had thus disappeared, the paradox could be obtained. Stimulating such a preparation with frequencies between the minimum frequency at which the slow contraction appears (we found this to be about 5 per second for *Cambarus* and about 2 per second for *A. trowbridgii*) and that at which the fast contraction appears (under these circumstances about 15 per second), the action currents on stimulation of the thick fiber are very large but no contraction results, those on stimulation of the thin fiber small but a distinct contraction occurs, increasing, of course, with the frequency (Fig. 2). In this case there is even in *Cambarus* often no facilitation at all of the fast action currents, whether a contraction is elicited or not, but sometimes some facilitation appears in those cases in which the action current had been considerably reduced during the stimulation series given to extinguish the fast twitch. In preparations in which the paradox can be obtained, contractions elicited by a low-frequency stimulation of the thick fiber are smaller than the corresponding contraction on stimulation of the thin axon.

TABLE 2*

Astacus trowbridgii: STIMULATION OF THE THICK FIBER OF THE ADDUCTOR

Number of Impulse	Height of the Mechanical Contraction (in Mm.)	Height of the Action Current (in Mm.)
2	>50	22 5
3	>50	22 5
60	>50	21.5
90	15	21.0
120	2	21 0
150	1	21.0
180	....	21.0
240	....	20.0

* One induction shock every 5 seconds.

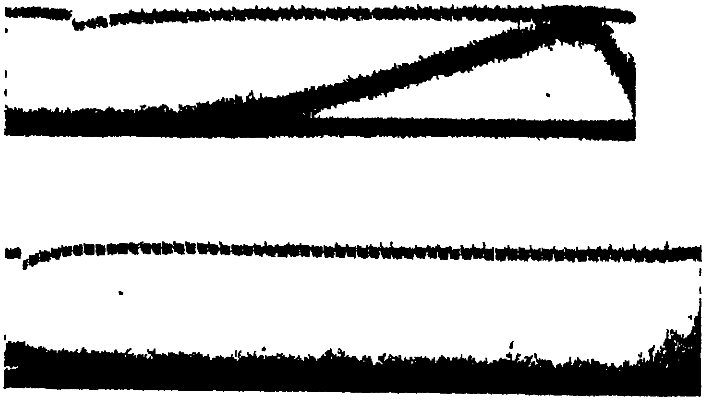


FIG. 2.—*Cambarus clarkii*, adductor muscle. Paradox with a stimulation frequency of 13 per sec. *A* and *B* are from one continuous record. *A*, thin axon stimulated, action currents cannot be distinguished from the current escape. *B*, thick fiber stimulated, no contraction, only relaxation of previous slow contraction, high action currents. No facilitation of the action currents, instead a decrease. Time mark is $\frac{1}{5}$ of a sec.

Another phenomenon which shows the relative independence of the mechanical contraction and the action currents is the following: When the fiber of the slow contraction is stimulated for a long time (frequency, e.g., 50 per second) an increase in the height of the action currents takes place in the beginning but is followed after a rather long period of constancy by a decrease. When the accompanying contraction is registered (strong isometric conditions) it is found that the contraction still increases long after the action currents have begun to diminish (see Table 3). Thus we find here that the mechanical contraction and the action current are influenced in opposite directions.

The fast action currents almost always diminish from the beginning; this reduction is especially quick with high frequencies, but here the mechanogram is much more affected by the fatigue and disappears long before the action currents are so small that they are no longer distinguishable.

TABLE 3*

Cambarus clarkii: FARADIC STIMULATION OF THE THIN FIBER OF THE ADDUCTOR

Time after Beginning of Stimulation (in Secs.)	Height of the Action Current (in Mm.)	Height of the Mechanical Response (in Mm.)
1	5.8	2.5
3	7.0	3.7
6	8.0	6.0
15	8.0	9.5
30	7.8	14.5
45	7.1	19.5
60	5.8	21.5
75	5.0	21.7
90	4.6	21.0

* Frequency 50 per second.

Investigating the slow and the fast contractions with high frequencies of stimulation, we observed that neither of them gave a normal response for any length of time (2 seconds) with frequencies above 200 per second. Nevertheless both showed contractions for short times on stimuli up to about 700 per second. Whereas the fast contraction increased in speed with the frequency, the slow contraction had a maximum speed at 200–400 per second.

It was of interest to note that in several preparations the shape of the fast contraction with a frequency of 50 per second was about the same as that of the slow contraction stimulated with 200 per second, both developing the same maximal tension (duration of stimulation 2 seconds).

EXPERIMENTS WITH *Cancer*

Whereas in *Blepharipoda* we were always able to obtain the paradox with fresh preparations and we have never failed to obtain it in *Cambarus* using the method described, it is rather astonishing that we have not been able to find the paradox in *Cancer anthonyi*. From the fact that in this animal the interval in which the fast contraction is lower than the slow one is relatively prolonged (Wiersma and van Harreveld, 1938), one would expect that on lowering the frequency the fast contraction would disappear first. On the contrary, we found that in fresh preparations stimulation frequencies so low that they do not elicit a slow contraction still cause a fast one. In relation to this it is of importance that the range of summation intervals of two induction shocks, which when applied to the thick fiber cause a mechanical response, is much larger in *Cancer* (2 to more than 36 σ) than in *Blepharipoda* (2–10 σ). In correspondence with this the stimulation of the thick fiber causes contractions on lower frequencies of stimulation than in *Blepharipoda* (10 per second still gave a distinct contraction). This may explain the absence of the paradox in *Cancer*. We noticed particularly that the tension developed by the fast contraction at these very low frequencies increased only during the first few stimuli and then remained constant. With somewhat higher frequencies the main development of the tension of contraction still occurs initially, and only a small increase takes place with continued stimulation. On the other hand, the slow contraction continues to grow for a longer time and attains higher tensions at stimulation frequencies below 100 per second.

DISCUSSION

It is now almost certain that both the fast and the slow contractions can be elicited in every muscle fiber of a muscle with a double motor innervation. The anatomical evidence points strongly in that direction, and recently we have obtained further evidence for this, observing muscle fibers of the adductor muscle of *Cambarus* contracting as well on stimulation of the axon for the fast contraction as on that of the slow contraction. We shall report on this later.

The facilitation of the action current has been referred by Katz (1936) to an increase in the number of muscle fibers reached by the nerve impulse in the course of a faradic stimulation. According to this explanation the action currents of the individual muscle fibers can follow the all-or-none relation and the difference between the slow and the fast contraction in *Cambarus* (e.g.) would be that, on stimulating the fiber for the fast contraction all the muscle fibers are excited and respond with an action current, whereas on stimulation of the fiber for the slow contraction only a few muscle fibers would be reached by the first nerve impulse, some more by the second, etc. We consider the "paradox" as a phenomenon excluding this explanation of the increase of the action current. Especially conclusive are the experiments on *Cambarus clarkii* and *A. trowbridgii*. In these animals it was observed that the action currents accompanying the fast contraction, whether they cause a mechanical response or not, are all of the same (maximal) height. This would, according to this explanation, show that approximately all the muscle fibers are excited, but in the "paradox" do not show a contraction. If now the slow contraction was due to the same mechanism, it is inexplicable why the small number of fibers excited during a stimulation with low frequency—as is indicated by the low action currents—should cause a mechanical response. Our conclusion must be that the action currents evoked by the stimulation of the two motor fibers are an index or accompaniment of activities which are located in different places in the same muscle fiber.

This conception excludes the possibility that the action currents in the individual muscle fibers follow the all-or-none relation in its strict sense, as in each muscle fiber can be produced action currents of at least two different heights. We do not believe that these two action currents are constant in amplitude or other character. We consider the action current of the crustacean muscle as the index, not of a conducted, but of a local, process (compare the conclusions of Rosenblueth, Davis, and Rempel, 1936, concerning the first electrical response of the nictitating membrane of the cat).

Both the action currents of the slow and those of the fast contractions show a distinct facilitation in marine crustaceans. This resemblance is further increased by the fact that both mechanisms show latent facilitation. It is thus likely that, although the two processes differ in their localization in the muscle fiber and in the readiness of their facilitation, they are fundamentally similar in character. In *Cambarus* the facilitation of the action currents of the fast contraction is usually absent. The same can be stated for the action currents in preparations in which the mechanical response to a single nerve impulse has disappeared. Since the action currents in the latter case do not show facilitation, it is evident that the appearance of a mechanical response when the stimulus is repeated with a certain frequency cannot be ascribed to a change in the action potentials. It must be assumed, therefore, that a second kind of facilitation is present, which must be referred to processes interposed between the action current and the contraction.

It is likely that the same kind of facilitation exists in the slow contraction. This cannot be demonstrated in the beginning of a stimulation, since here a marked increase of the action currents by the facilitation is seen, and this effect might be chiefly responsible for the increase in the mechanical response. It was mentioned, however, that after some time the action currents start to decrease again (fatigue), whereas at the same time the mechanical contraction still continues to increase. Evidently this increase in the mechanical contraction is not caused by an increase of the action potentials but is to be referred to some other process of facilitation, located between the action current and the contraction.

We conclude that although the different steps in the transmission from the nerve impulse to the muscle fiber resemble each other closely in the slow and in the fast contraction, these processes must take place in different situations on the muscle fiber and must also show differences in their relative magnitudes.

SUMMARY

It is shown that on stimulation of the thick and the thin nerve fiber, innervating the adductor muscle of the claw of *Blepharipoda*, a range of frequencies can be found in which the thick axon gives large muscle action currents and no contraction, whereas the thin axon gives very small action currents but a good contraction. This paradoxical phenomenon can also be obtained in the adductor of the claw of *Cambarus*, when the twitch caused by a single nerve impulse in the thick fiber has disappeared. It was not obtained in the adductor of the dactylopodite of the walking leg of *Cancer*.

From this and other experiments the tentative conclusion is drawn that the fast and the slow contractions take place in the same muscle fibers, each having its own mechanism of transmission between nerve impulse and contraction. These may be in both cases qualitatively the same but quantitatively different.

LITERATURE CITED

KATZ, B. 1936. Jour. Physiol., 87:199.
ROSENBLUETH, A.; DAVIS, M.; and REMPREL, B. 1936. Amer. Physiol, 116:387.
VAN HARREVELD, A., and WIERSMA, C. A. G. 1936. Jour. Physiol., 88:78.
WIERSMA, C. A. G. 1933. Z. vergl. Physiol., 19:349.
———. 1937. Z. vergl. Physiol, 24:381.
WIERSMA, C. A. G., and VAN HARREVELD, A. 1938. Jour. Exper. Biol., 15. (In press.)

THE RESPONSE TO MECHANICAL SHOCK BY THE CERCARIAE OF BUCEPHALUS ELEGANS[1]

(One figure)

HARRY T. FOLGER AND LLOYD E. ALEXANDER
Fisk University

MECHANICAL shock has long been known to have a pronounced stimulating effect on many organisms, eliciting responses in some instances that are as clear cut and precise as those brought about by such agents as light or electricity But, owing largely no doubt to the difficulty of controlling the magnitude of the stimulus with any degree of certainty, there is a dearth of exact knowledge concerning the effects of mechanical shock. Numerous observations have been recorded to be sure, but most of them have been merely incidental and have noted little more than that the organisms under consideration do respond to such a stimulus.

However, a few observations of a more detailed nature have been made. Several workers have noticed the suspension of movement that results when streaming cells of various plants are subjected to mechanical shock. Ewart (1903) found the protoplasmic streaming not only stops when cells of *Chara* and *Nitella* are so stimulated but that the time from the application of the shock until stoppage occurs—the reaction time—is longer after a light shock than after a heavy one and that movement remains in abeyance longer under the latter condition. Folger (1926) has reported that mechanical shock causes a cessation of protoplasmic flow in *Amoeba proteus* and that the same thing obtains with regard to the reaction time and period of quiescence as in streaming cells of *Chara*. Moreover, he found that a distinct refractory period follows the reaction. A certain amount of time must be allowed for recovery from the effects of a previous shock before the amoeba will respond to another, and a recovery which is not quite complete results in a reaction time that is longer and a period of quiescence that is shorter than after complete recovery.

Though there has been little or no disagreement in the observations on the effect of mechanical shock, the meagerness of evidence makes the study of other forms desirable. Most organisms are not suitable for work of this sort, as either they do not react at all or, if they do, the extent of the reaction is not easy to measure. The discovery of an animal, the cercaria of a flatworm parasite, which not only responds readily and consistently but with a reaction that can be accurately timed with a stop watch, has led to the investigation which is to be presented.

MATERIALS AND METHODS

The animals that were used in the following experiments were the cercariae of the gasterostome parasite, *Bucephalus elegans*, larval stages of which are passed in the gonad of the fresh-water clam, *Eurynia iris*. The cercariae emerge through the excurrent siphon of the clam and execute active swimming movements.

---

[1] Contribution from the Biology Laboratory of Fisk University The authors are indebted to Dr. Arthur E. Woodhead for help and advice in the securing of experimental animals.

A large number of clams was collected in the early fall from the Huron River near Ann Arbor, Michigan. Infected individuals were isolated and kept in a cool room, in a large concrete tank, or imbedded in sand in glass aquaria. When animals were desired for experimentation, a few of these clams were transferred to finger bowls and put in a warm room. Within 24 hours there was usually an abundance of cercariae swimming about in the bowl.

A drawing of a cercaria is shown in Figure 1. As may be seen, it is of the fork-tailed type, the tail being cleft for almost its entire length. The two rami resulting from the bifurcation constitute the bulk of the animal. Locomotion is brought about by movements of the rami, which become extended so as to form a straight line and then curl up and at the same time shorten and thicken, assuming the position shown in Figure 1. When fully extended, the cercariae are from ½–1 cm. in length. The rate of contraction and the extension of the rami depends on several factors, among them temperature and age, and there is considerable individual variation. At about 20° C. the rate is usually between 55 and 70 contractions per minute. A stimulus such as sudden illumination or mechanical shock causes a temporary cessation of movement.

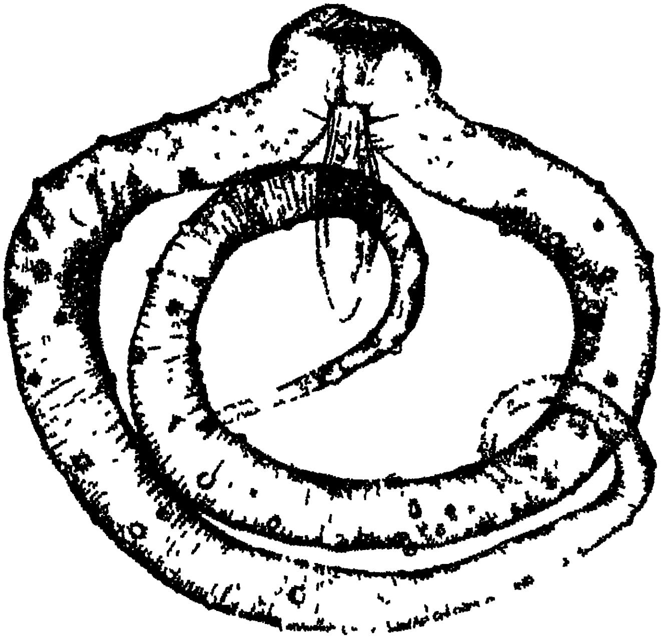


Fig 1

The precise nature of the reaction to mechanical shock depends upon the intensity of the stimulus. If the shock is slight, it may have no effect, or it may cause the cercaria to cease progression through the water and the rami to undergo quivering motions, but without complete suspension of movement. A somewhat heavier shock causes the animal to become quiet, and in doing so the rami are generally folded. Very heavy shocks not only bring about cessation of movement but may cause the rami to remain in any position, extended or contracted. In every instance in which movement stops, there is an appreciable reaction time, which, as used in this paper, consists of the time from the reception of the stimulus until the cessation of motion, without regard to the position of the rami.

The apparatus which was used in the experiments consisted of a narrow glass tube, 12.25 inches in length, supported in an upright position on a ring stand, and weights of the various sizes indicated in the text, made by cutting off appropriate lengths from wire nails. These weights, when dropped through the glass tube upon the deep depression slide on which the cercariae were put, brought about the shock. The slide was placed flat on the base of the ring stand in such a position that the falling weights would strike it midway between depression and end. The glass tube was clamped loosely near the top, with the lower part hanging freely, and the lower end almost touching the slide. On impact, the weight rebounded up into the tube, and a quick push against the latter threw it to one side, thus preventing it from again striking the slide and adding to the shock already produced. Care was taken in every test to make certain that the cercaria was swimming freely in the water and was not in contact with the glass. Time was measured with a stop watch. The reactions were sufficiently sharp to permit timing to one-fifth of a second.

EXPERIMENTAL RESULTS

Since *Bucephalus* cercaria react to mechanical shock after an appreciable reaction time, an obvious approach to a better understanding of the effect of this stimulating agent is an investigation of the relation existing between the intensity of the mechanical shock and the duration of the reaction time, for, as we shall see, the latter varies with the magnitude of the stimulus. The results of such an investigation are shown in Table 1. In this table are recorded the reaction times of 13 individuals, stimulated by shocks brought about by the fall of weights ranging in size from 177 mg. to 2,525 mg. Since, as already stated, each dropped a distance of 12.25 inches, the shock was proprotional to the size of the weight. So far as practical use is concerned, the foregoing weights represent extremes. It is shown in the table that only 2 animals out of 13 responded to the 177-mg. weight; a lighter one would have been even less effective. And a weight of only a little more than 2,525 mg. not only caused such a jar as to endanger the glass slide but often broke off the rami of the cercariae.

The behavior of individual No. 1 may be presented as a typical example illustrating the relation between intensity of shock and reaction time. In this animal the 2,525-mg. weight brought about a reaction in 0.8 seconds, the 2,025-mg. weight in 1.2 seconds, the 1,750-mg. weight in 1.2 seconds, the 1,040-mg. weight in 1.4 seconds, the 631-mg. weight in 2.4 seconds, the 501-mg. weight in 2.0 seconds. There were no responses to weights of 288 mg. and 177 mg.

Although not denoted in every instance, it is clear from this example that the reaction time tends to increase with a decrease in the force of the mechanical shock. Exactly the same thing is indicated in the reactions of the remaining 12 individuals, and, as shown in the lower row of Table 1, it is indicated especially clearly in the average reaction times of all of the animals. These averages, brought about by the fall of weights from the heaviest to the lightest, respectively, were 1.19, 1.44, 1.65, 1.70, 2.17, 4.38, 5.90, and 5.50 seconds. The decrease in the final instance is probably of no significance, as all except 2 of the animals failed to respond to so slight a stimulus.

While the behavior just described is certainly generally true, it is only fair to note certain exceptions. The reactions of individual No. 4 were obviously exceptional. This animal reacted to a weight 2,525 mg. in 1.8 seconds, to a weight of 2,045 mg. in 2.2 seconds, and to a weight of 1,750 mg. in 3.0 seconds. So far the reactions are as one would expect; that is, the reaction time increased with decrease in intensity of stimulation. However, stimulation by a still lesser weight, one of 1,040 mg., not only was not followed by a still further increase in reaction time but actually by a very decided decrease, the reaction time dropping to 1.0 second. Further reduction in the size of weights brought about the customary increase in reaction time. The weight of 631 mg. caused a reaction in 1.2 seconds, and that of 501 mg. in 1.4 seconds. Folger (1925) noted similar sudden changes in the responses of *Amoeba* to light. Such irregularities in reaction are due to changes in the physiological state of the organism and by no means invalidate a conclusion, such as the one proposed in the first sentence of the preceding paragraph, which presupposes that the physiological state will remain constant.

TABLE 1*

SHOWING THE REACTION TIME FOLLOWING SHOCKS OF DIFFERENT DEGREES OF MAGNITUDE

ANIMAL NO.	WEIGHT IN MILLIGRAMS							
	2,525	2,045	1,750	1,040	631	501	288	177
1	0.8	1 2	1.2	1.4	2.4	2 0	No	No
2	1.0	1.4	1.0	2.0	1.4	2 0	5.0	No
3	1.2	2.0	1.4	1 4	2.0	1.4	8.0	5.0
4	1 8	2.2	3.0	1.0	1.2	1.4	No	No
5	1.0	1.0	1.0	1.2	2.0	1.8	No	No
6	1.0	1.4	1.4	2.0	1.2	15.0	No	No
7	1.0	1.2	1.2	1.2	3.0	No	No	No
8	1.4	1 0	1.2	1.4	2.0	2.0	No	No
9	1.0	1.0	1.4	1.4	3.4	3.6	No	No
10	1.0	2.0	2.0	2.0	No	No	No	No
11	1.2	1.4	1.6	2.0	3.0	4.4	4.4	6.0
12	2.0	2.2	3.0	3.0	2.0	12 0	8.0	No
13	1.0	1.0	2.0	2.2	2.4	2.6	4.0	No
Average	1.19	1.44	1.65	1.70	2.17	4.38	5.90	5.50

* The reaction time consists of the time from the application of the stimulus until the cercaria ceased to move. *No* indicates failure to respond. At least 3½ minutes intervened between tests.

Doubtless many factors besides intensity of shock affect the reaction time, but only one other has been investigated—the effect which a given shock has on the reaction to another shock which follows quickly after it. The results of this study are set forth in Table 2. Nineteen different animals were used in the experiment, the procedure of which consisted simply in applying a series of stimulations to the chosen animals, with certain intervals, ranging from 60 seconds to 15 seconds, between them. As indicated in the table, animals Nos. 1 and 3 were subjected to nine successive shocks, with intervening periods of 50 seconds. Both animals reacted in every instance, and in both the reaction time remained practically constant, at about 1.2 seconds. Number 2 gave exactly the same results up to the sixth reaction, when it was accidentally killed. Animals 4, 5, 6, and 7 were treated as the preceding animals, except that intervals of 30 seconds were allowed between shocks. Numbers 4, 5, and 7 continued to respond for nine or ten times without an appreciable change in reaction time. They would probably have responded for a longer time had they received further stimulations. Number 6 failed to respond to the third shock. Animals 8, 9, and 10 were allowed 25 seconds between shocks. Number 8 failed to respond after the third stimulation, No. 9 after the fourth stimulation, and No. 10 after the sixth stimulation. And not only was there finally a cessation of response but the reaction time decreased very considerably before the responses stopped. Thus, at the first shock the reaction time of cercaria No. 8 was 1.2 seconds, but at the third shock it was only 0.8 seconds. The reaction time of No. 9 fell from 2.0 seconds at the first stimulation to 0.8 seconds at the third. Cercaria No. 10 reacted to six successive shocks, with reaction times expressed in seconds as follows: 1.6, 1.0, 1.0, 0.8, and 0.6. The next group, consisting of animals Nos. 11, 12, and 13, were allowed 20 seconds between stimulations. The first two ceased to respond after the fourth shock, the third after the fifth shock. The average reaction times of the 3 animals amounted the 1.3 .seconds, 0.9 second, 0.7 second, and 0.7 second for four successive stimulations. The final group of 6 animals were allowed 15 seconds between shocks. None of them responded more than three times, and No. 19 only twice. The average reaction times were 1.8 seconds, 1.3 seconds, and 1.0 second.

TABLE 2*

SHOWING EFFECT OF INCOMPLETE RECOVERY ON THE REACTION TIME

Reaction No.	Time Allowed for Recovery																							
	60 Sec.				30 Sec.					25 Sec.				20 Sec.				15 Sec.						
	Animal No.			Ave.	Animal No.				Ave.	Animal No.			Ave.	Animal No.			Ave.	Animal No.						Ave.
	1	2	3		4	5	6	7		8	9	10		11	12	13		14	15	16	17	18	19	
1.....	1 0	1.2	1.4	1.2	1.4	1.0	1.0	1 0	1.0	1 2	2.0	1.6	1 6	1.6	1.2	1.0	1.3	1.6	1.4	2.0	1.4	1.4	1.0	1.8
2.....	1.2	1.2	1.0	1.1	1.4	1.2	1.0	1.0	1.2	1.2	1.0	1.0	1.1	1.0	0.8	1.0	0.9	1.0	1.4	1.4	1.0	0.8	0.8	1.3
3.....	1 0	1.2	1.4	1.2	1.4	1.0	No	1.0	1.1	0.8	0.8	1.0	0.9	0 8	0.6	0.8	0 7	1 0	1.2	1.2	0.8	1.0	No	1.0
4.....	1.0	1.2	1.2	1.1	1.4	1.0		1.0	1.1	No	0.8	0.8		0.6	0.6	0.8	0.7	No	No	No	No	No		
5.....	2.0	1.0	1.4	1.5	1.4	1.0		1.0	1.1		No	0.8		No	No									
6.....	1.0	1.2	1.4	1.2	2.0	1.0		1.0	1.3			0.6												
7.....	2.0		1.2	1.6	1.4	1.0		1.0	1.3			No												
8.....	1.0		1.2	1.1	1.4	1.0		1.2	1.2															
9.....	1.0		1.2	1.1	1.2	2.0		1.2	1.5															
10....					1.4																			

* Reaction times given in vertical columns and expressed in seconds. Note that 30 seconds was sufficient for complete recovery, as indicated by a reaction time that remained constant after repeated tests, and that 25 seconds or less did not permit complete recovery, as indicated either by a reaction time that was shorter than usual or by failure to respond.

From these results it is apparent that at least 30 seconds are necessary for complete recovery from the effects of a mechanical shock and that incomplete recovery is made evident either by failure to respond to another shock or by a reaction time that is shorter than that after complete recovery. Furthermore, there is clearly a cumulative effect. A series of stimulations, with an insufficient recovery period between, results in a steady decrease of the reaction time until finally there is no reaction at all.

DISCUSSION

The increase in reaction time with decrease in the force of the mechanical shock that has just been observed in the reactions of cercariae is exactly what one would have expected. As mentioned in the introduction, this stimulating agent is known to have a similar effect on some other organisms, and the same thing is true in the reactions of various organisms, including the cercariae of *B. elegans* (Bevelander, 1933),[2] to other forms of stimuli, notably light. But the result of insufficient time for recovery is rather surprising. A number of investigators have observed that such a condition may have a very decided effect on the reaction time, but in nearly every instance, both in the case of mechanical shock and in the case of light, the effect has been manifested by an increase in reaction time. Here there is just as decidedly a decrease. Just why *Bucephalus* cercariae should behave differently from other organisms in this one respect is not certain, especially as their reactions are in accord in other respects. It is possible, however, that they do not behave so differently as appearances indicate.

A well-known fact of physiology is the temporary loss of sensitivity which follows stimulation of a nerve, giving rise to the interval known as the refractory period. This period is divisible into two parts—the absolute refractory period, which follows immediately after stimulation and during which there is a complete loss of excitability, and the relative refractory period, during which the nerve will respond to stimulation but not so readily as ordinarily. It is a significant fact that distinct refractory periods, both absolute and relative, are known to follow the reactions of a number of organisms to light and mechanical shock. *Amoeba*, for example, responds to both of these stimulating agents by a cessation of movement. It will not immediately respond to another stimulus at all; and, even after it does begin to respond, it is at first less sensitive than usual, as indicated by an increase in the reaction time. But another thing which has been observed in nerve is that at the beginning of the relative refractory period there is a supernormal phase, during which the nerve is not less but more excitable than usual. Folger (1925) has observed a supernormal phase in the response of *Amoeba* to light. While feeding, an amoeba refuses to respond to light at all. A minute or so after it has fed, it is considerably less sensitive than usual, but immediately after feeding it responds much more readily than at any other time. It appears not at all improbable that the increase in sensitivity in the cercariae of *B. elegans*, which is caused by repeated shocks with only a small time interval between and made evident by a decrease in reaction time, is similar to the supernormal phase in the response to light which follows feeding in *Amoeba*.

SUMMARY

1. The cercariae of *B. elegans* react to mechanical shock by a cessation of movement, which takes place after an appreciable reaction time.

2. The reaction time varies inversely with the magnitude of the shock. This agrees with the reactions of various other organisms to both mechanical shock and light.

---

[2] In response to light the reaction time increases with decrease in luminous intensity.

3. After cercaria has reacted to a mechanical shock, it will not immediately react to another. About 30 seconds are required for complete recovery from the effects of such a stimulation. Recovery may be partial, as indicated by a reaction time that is less than that obtained after complete recovery. This does not agree with the reactions of other organisms, where incomplete recovery from the effects of mechanical shock or light is indicated by an increase in reaction time.

LITERATURE CITED

Bevelander, G. 1933. Response to light in the cercariae of *Bucephalus elegans*. Physiol. Zoöl., 6:289–305.

Ewart, A. T. 1903. On the physics and chemistry of protoplasmic streaming in plants. London: Oxford Press. Pp. 131.

Folger, H. T. 1925. A quantitative study of reactions to light in amoeba. Jour. Exper. Zoöl., 41:261–90.

———. 1926. The effects of mechanical shock on locomotion in Amoeba proteus. Jour. Morph. and Physiol., 42:359–70.

THE NUTRITIONAL REQUIREMENTS OF BLATTELA GERMANICA

(Three figures)

C. M. McCAY
Animal Nutrition Laboratory, Cornell University, Ithaca

IN SPITE of the wealth of available material, insect nutrition has been given little attention in contrast to that of such vertebrates as the rat.

Trager (1935) has recently summarized the evidence showing that a number of insect species have specific requirements for vitamin-like compounds found in such materials as yeast and wheat germ. However these substances seem to differ from B_1 and lactoflavine although some insects seem to require some such fractions. Trager finds mosquito larvae need fractions found in liver extract and yeast products.

The webbing clothes moth also requires part or all of the B-complex for growth and development (Crowell and McCay, 1937).

The cockroach (*Blattela germanica*) has no vitamin requirements according to the findings of Wollman (1926). This author generalized upon his findings, claiming that insects are distinguished from the higher animals in their ability to grow and reproduce without such accessory factors. Zabinski (1929) also studied the requirements of this insect and concluded they required no other source of nitrogen than glycine.

These remarkable claims for an insect that is an unwelcome guest in so many nutrition laboratories made this ancient pest especially attractive for study.

BREEDING AND REARING A STOCK OF COCKROACHES

For a period of several years various cages were tried for nutrition experiments with cockroaches. The design shown in Figure 1 has proved very satisfactory both for testing a given diet upon isolated individuals and for studies with groups. In Figure 2 is shown a satisfactory breeding cage which is used to provide an ample supply of stock animals.

The test cage of Figure 1 is a half-pint milk bottle fitted with a three-hole cork stopper. Three tubes of 0.9 cm. glass tubing provide an air hole, access to a vial containing a piece of cotton soaked in water, and another vial holding the diet to be tested. The food can also be placed in the bottom but it seems more liable to attacks by molds. Numerous studies in the course of the last six years have indicated that this cage provides adequate ventilation and nearly optimum humidity. Among the types of cages tested have been those constructed of very fine wire screening and open on all sides. The humidity was controlled in such cages. These have little advantage and many disadvantages. Other studies have been made with several vents in the cork stopper of the milk bottle. All of these have indicated that the cage of Figure 1 is probably the most satisfactory devised thus far.

For producing large numbers of stock animals the museum jars of Figure 2 have proved useful. The water in such cages is provided by a funnel filled with a cotton plug and set in a beaker of water. This prevents the drowning of the insects and fouling of the water. The water is easily renewed by pouring from a wash bottle at intervals of several days. The food supply is placed in a vial or a small beaker.

For six years a stock diet of equal parts of dry skim milk and ground whole wheat has proved adequate for the entire life-cycle of the species. From time to time various other food substances have been tried but no additional advantage has been found.

For rapid growth and reproduction a temperature of twenty-five degrees or more is desirable. For testing foodstuffs an old refrigerator equipped with a thermostat has proved very satisfactory in providing a constant temperature.

The growth rate of this species is similar to that of the rat. It is easily followed by periodical weighings upon an analytical balance since the range of weight from the young to the largest adult varies between one and a hundred milligrams. This insect is very sensitive to ether anesthesia. This provides a convenient method of handling.

For securing test animals the first step is the isolation of females bearing egg sacs. This is done by moistening the cloth cover of the stock cage with ether. The females are then placed in test bottles using a pair of light forceps. These are examined at daily intervals. The date of hatching of the young is recorded. They can then be placed under light ether anesthesia and distributed to test bottles. Ten individuals per bottle afford a very satisfactory group. Special studies have shown that this anesthesia does not modify the growth-rate even if repeated at weekly intervals.

The productivity of the females can be illustrated by a single experiment. Three females bearing egg sacs were placed in a stock cage. Five months later this cage contained seventy adults, many of them bearing mature capsules. This suggests the possibility of using this species for population studies with results that might be more applicable to higher animals than those made with *drosophila*, since the food habits are more nearly like those of vertebrates.

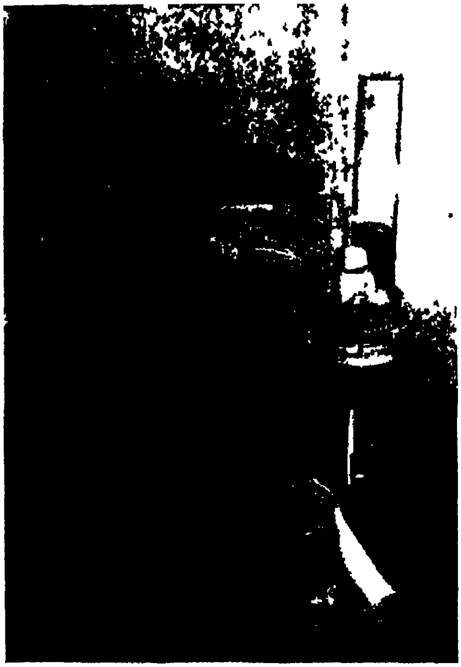


FIG 1.—Cage for nutrition studies with cockroaches. Food can be placed either in the vial on the sidetube or in the bottom The upright vial has a cotton plug saturated with water. The cloth-covered tube provides air.

THE CONVERSION OF ELEMENTS FROM FOODSTUFFS INTO BODY TISSUES BY INSECTS

Various species differ widely in their ability to convert the chemical elements of foodstuffs into body tissue or secretions. Lusk (1928) notes that the cow can secrete 5,378 calories of energy in the form of milk, while its body burns 11,464 calories as measured by the calorimeter. Armsby and Moulton (1925) has discussed the numerous variables, such as the rate of growth, individual variability, and species differences that determine the efficiency of higher species in building the chemical elements of foodstuffs into body tissues. From the data of Palmer and Kennedy (1931) rats in the course of their growth seem able to convert about 30 per cent of the nitrogen of their food into that of their body. The accumulation of errors in such a calculation may amount to as much as 30 per cent of the above value.

A few studies of the ability of lower vertebrates to convert foodstuffs into body tissues indicate an efficiency superior to the best of the higher vertebrates. Brook trout are somewhat superior to such species as swine or chickens (McCay, 1937). In some time periods of 4 weeks trout may even require less than a gram of dry foodstuff to produce a gain of 1 gram in live weight. Poikilothermes may have some advantage in the conversion of foodstuffs, since they have no body temperature to maintain.

FIG. 2.—Stock-breeding cages. The one above is ready for use while the one below contains insects. The funnel in the beaker of water is stuffed with moist cotton. The feed is in the vial. The cloth covering moistened with ether provides the anesthetic when females are removed.

Since no data are available concerning the conversion of elements by insects, the present study was undertaken. Conditions were favorable for such a determination since a satisfactory dry diet had been developed that permitted an insect to pass through its entire life-cycle without limitations due to the quality of the diet. The use of a single diet may permit an accurate estimate of the utilization of calories, but the determination of the efficiency of the conversion of other elements in such a diet cannot be optimum unless such elements are present in the diet at the lowest levels that just permit optimum growth. There is no reason to believe that this was the case in the present study. For this reason the values obtained are accurate for the diet under consideration but are not optimum ones except in the case of the calories concerned.

The efficiency of transformation of chemical constitutents into body tissues can be measured in *B. germanica* by weighing a known allowance of foodstuff, allowing groups to grow to maturity upon it until all the foodstuff is consumed, and finally analyzing the bodies of the insects produced upon the foodstuff. The greatest error in this procedure is due to the foodstuff retained in the intestinal tract. This structure is so delicate that it is impossible to wash it free from food residues in extensive numbers of individuals.

The following procedure was employed: Three groups of young insects, one to two days after hatching, were confined in separate jars after their body weights were recorded and a sufficient number had been taken for analysis. Into each jar was placed a vial of foodstuff that had been carefully weighed. When the insects in each jar had attained adult size they were removed, weighed, and preserved for analysis. Into each jar a new group of young was introduced and the procedure repeated until all the foodstuff was consumed.

From this study the weight and composition of both the foodstuff and the insects produced were determined by analysis. In the preliminary preparation the foodstuff was carefully moistened with 95 per cent alcohol and then dried carefully *in vacuo*. A sample was sealed for analysis. The foodstuff consisted of a mixture of equal parts of dried skim milk and whole ground wheat.

Each jar produced nearly three generations of mature insects. The following analytical procedures were employed: nitrogen—Barrett and Jones (1932); ash—ignition in the muffle furnace; calcium—Morris, Nelson, and Palmer (1931); phosphorous—Youngberg and Youngberg (1930); lipids—extraction of the dried material with ethyl ether in the Soxhlet apparatus for 16 hours; I. No.—Yasuda (1931).

In order to reduce the error for the food residues of the intestinal tract to a minimum, the insects were fasted for 24 hours before killing in each case.

In order to determine the error that could be expected from the food residues, groups of adult insects not involved in these studies were killed without fasting and also after intervals of 1–5 days without food. The longest period may cause death if individuals are isolated and cannibalism if they are associated. Fifty representative adults from each group were weighed, the posterior end of the body snipped, and the intestinal tract removed by pulling the head. This tract was then removed. The tracts and contents were dried and weighed separately from the bodies. With no period of fasting the tract and residues were equal to 9 per cent of the body weight while after 5 days of fasting this value fell to 4.6 per cent. In the latter case there was little food residue and this probably represents the intestinal tract. The error from neglecting food residues in insects that have fasted for one day must then be less than 5 per cent.

The analytical data from these studies are assembled in Tables 1 and 2. These data indicate clearly that the insect is very efficient in the conversion of foodstuffs compared to higher animals. This species is able to build nearly a unit weight of the living body from a unit of dry foodstuff. It is able to synthesize fat since the bodies of the insects contained about three times as much lipid as the foodstuff, ingested. Also, this body fat had only about half the iodine number of that ingested, which affords evidence for a characteristic body fat.

In these experiments about 60 per cent of the nitrogen, 10 per cent of the calcium and 25 per cent of the phosphorus of the diet were converted into body tissue. Analytical values are not available for the chitin of the insect and the carbohydrate of the diet, but estimates indicate that about 40 per cent of the calories in the diet were built into the body of the insect.

THE FEEDING OF MIXTURES OF PURIFIED CONSTITUENTS

In preliminary trials it was found that this species could not grow upon mixtures of casein, starch, cellulose, sugar, lard, and mineral mixture unless this mixture were supplemented with yeast.

TABLE 1

THE CONVERSION OF FOODSTUFF INTO BODY TISSUE BY INSECTS

Expt. No.	Wt. Insects Added (Grams)	Wt. Insects Removed (Grams)	Dry Wt. Insects Removed (Grams)	Per Cent H_2O	Calcd. Dry Wt. Increase (Grams)	Dry Food Consumed (Grams)
M-1	0 5002	8 8483	3 1359	64 6	2.9587	9 3418
M-2	0.6026	7 8599	2 8077	64 3	2 5922	8 2832
M-3	0 8766	8 8296	2.9789	66 3	2 6835	8.5769

TABLE 2

COMPOSITION OF THE INSECTS AND FOODSTUFF IN PERCENTAGE OF DRY WEIGHT

Expt. No.	N	Ash	P	Ca	Ether Sol.	I. No. of Fat
M-1	8.86	4.27	0 67	0 26	15 6	57
M-2	8 65	4.56	0 66	0.25	16.2	55
M-3	8 82	4.60	0 72	0.36	17.1	57
Foodstuff	4.48	4.63	0.80	0.72	0.86	111

This indicated there was a need for supplementary factors (McCay, 1933). In one study a group was maintained for 2 months upon the synthetic diet without supplements and then grew normally when transferred to the stock diet. This indicated that growth could be resumed after a period of retardation.

In order to check the first findings the diets shown in Table 3 were designed. The salt mixture (S.M.) was that of Osborne and Mendel (1919). The other constituents had been extracted for use in vitamin A testing with rats.

This experiment was started by placing twelve individuals in each bottle with the respective diets. The mean weight at the time they were placed in the bottles was 3.8 mg. The bottles were then placed in the incubator at 25° C. for 60 days. They were examined at intervals of 2 weeks and the growth noted, but they were not disturbed. At the end of 60 days they were etherized, weighed, and pasted to cards. The photograph, Figure 3, shows the relative sizes of the members of the different groups. In this case the differences are so obvious that it is hardly necessary to refer to the column of mean weights in Table 3.

In addition to confirming the first conclusions that cockroaches need some vitamin supplements, these data indicated some interesting lines of attack. In the first place, Group 20 was inferior to Group 14. These groups were not strictly comparable, however, since the diets differ in composition, but they suggest that gelatin may be less satisfactory than casein as a source of protein This problem is subject to solution with insects as well as rats but is difficult, because the cockroach can probably select yeast cells from the remainder of the food while the rat cannot This makes it necessary to work with soluble vitamin extracts instead of particles so that no opportunity is afforded for selection by the insect. It is quite possible that gelatin may be an incomplete protein in Diet 20 and that there may be essential amino acids for insects as well as rats. This experiment might easily fail to show this if the insect ate enough yeast cells to satisfy its amino acid requirement.

THE COMPOSITION OF DIETS WITH THE FINAL NUMBER ALIVE AND THE MEAN WEIGHT

Diet No	Composition	Number Alive after 60 Days	Final Mean Weight in Mg after 60 Days
5	Whole wheat 50, dry skim milk 50	10	lost
10	Casein 5, S M 5, sucrose 90	4	10 3
12	Casein 40, S M 5, sucrose 55	7	12 7
13	Casein 40, S M 5, starch 55	7	13 4
14	Casein 40, S M 5, starch 45, yeast 5, C L O 5	7	62 0
15	Casein 40, S M. 2, starch 58	8	14 3
16	Zein 20, S M 5, sugar 75	8	13 7
17	, S M 5, sucrose 95	11	7 4
18	Gelatin 5, S M 5, starch 90	10	13 8
19	Gelatin 20, S M 5, starch 75	8	12 7
20	Gelatin 20, S M 5, sucrose 65, yeast 5, C L O 5	9	45 6

These data indicate that Diet 17 was the most effective in preventing growth. In 60 days this group only grew as much as they would ordinarily grow in 4 days on a complete diet. On the other hand, a large percentage of this group survived

Unfortunately, the mean weight of the group fed the stock diet of wheat and skim milk was lost, but during the entire experiment this group grew more rapidly than any other. This mixture of natural foodstuffs is superior to the synthetic diet with its addition of cod liver oil and yeast. In all experiments upon this stock diet with its base of whole wheat it has been noted that the cockroach refuses to ingest much of the cellulose. This is sorted out and left behind as the diet is consumed.

These experiments also indicate that cannibalism will not interfere seriously with assay experiments in this case Even Group 10, which ended with only a few insects, made little gain in weight.

THE ACCESSORY FACTORS RESPONSIBLE FOR GROWTH

In a second series of experiments the earlier results were checked with modifications. New diets were devised. The composition of these diets is shown in Table 4. The constituents of these diets were purified in the same manner as those used previously Unfortunately, a number of other experiments in this series were lost through an accident

Diets 26, 27, and 28 were designed to determine if the fat-soluble vitamins alone were sufficient for growth Since Diet 26 gave poor growth compared to 27 and 28, this furnishes additional evidence that some factor in yeast is essential On the other hand, Diet 37 was satisfactory for growth although it contained little fat while 38, which had ample fat but no yeast, failed. This experiment is of special interest because the diet containing the lard tended to mold but this mold was not used in supplying a growth factor. Number 37 indicates that vitamin studies with this species can be made with diets low in fat. This is especially desirable because of mold growths upon fat-rich diets.

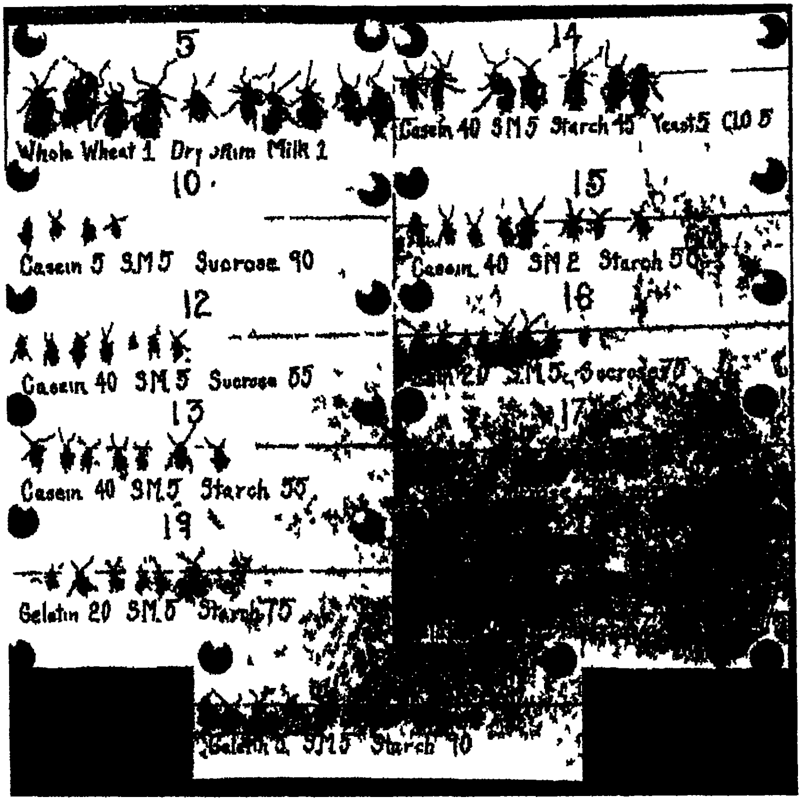

5
Whole Wheat 1 Dry skim Milk 1
14
Casein 40 SM 5 Starch 45 Yeast 5 CLO 5
10
Casein 5 SM 5 Sucrose 90
15
Casein 40 SM 2 Starch 50
12
Casein 40 SM 5 Sucrose 55
18
13
Casein 40 SM 5 Starch 55
17
19
Gelatin 20 SM 5 Starch 75
SM 5 Starch 70

Fig 3 —Cockroaches of the same age after being fed various diets for sixty days

Diets 39, 40, and 41 represent another attempt to determine if the quality of the protein is of importance to an insect. Owing to difficulties with molds this must be repeated, but this preliminary trial indicated that casein was the most satisfactory. In these experiments an alcoholic extract of yeast was used to avoid the danger of protein selection from yeast cells. Of the three proteins tested, casein was better than gelatin and this in turn proved better than zein. These experiments also provide a starting-point for determining which B fraction is essential for the cockroach. The alcoholic extract permitted good growth, but this experiment was not carried through the entire cycle to include reproduction.

The combined evidence from all these experiments indicates that this species has little if any need for vitamins A or D but that there is a requirement for some of the factors of yeast. In no case was growth upon synthetic diets equal to that upon mixtures of dried skim milk and wheat.

An additional series of sixty experiments that will not be reported in detail indicated that extracts of yeast made with 95 per cent alcohol were more potent as supplements than either water extracts or those of water-alcohol mixtures.

TABLE 4

DIETS VARYING IN THEIR CONTENT OF YEAST AND IN THE QUALITY OF PROTEIN

Diet No.	Composition	Growth Rate
26	Casein 20, starch 62, S. M. 3, lard 10, yeast 0, C.L.O. 5	poor
27	Casein 20, starch 59, S. M. 3, lard 10, yeast 3, C.L.O. 5	good
28	Casein 20, starch 55, S.M. 3, lard 10, yeast 6, C.L.O. 5	good
37	Casein 20, starch 72, S. M. 3, lard 0, yeast 5, C.L.O. 0	good
38	Casein 20, starch 67, S. M. 3, lard 10, yeast 0, C.L.O. 0	poor
39	Zein 20, starch 62, S. M. 3, lard 10 (Alc. Ext.), C.L.O. 5	poor
40	Gelatin 20, starch 62, S. M. 3, lard 10 (Alc. Ext.), C.L.O. 5	moderate
41	Casein 20, starch 62, S. M. 3, lard 10 (Alc. Ext.), C.L.O. 5	good

ATTEMPT TO ASSAY ALCOHOLIC EXTRACTS OF YEAST

An attempt was now made to assay the alcoholic extract of yeast. Five hundred grams of dry yeast were extracted in a continuous extractor on the steam bath with 2,500 cc. of 95 per cent alcohol. This extract was stored in a dark bottle in the refrigerator.

For a basal test diet a mixture of casein 30, salt mixture 4, and starch 66 was used. (The salt mixture was that of Osborne and Mendel.) Five grams of this diet were prepared for feeding by sprinkling a given amount of the alcoholic extract over it and evaporating it dry *in vacuo* at 65° C. This was then ground in a mortar to a powder.

Three bottles of ten insects each were used for the first three diets and eight bottles of ten each for No. 104.

The mean weight of the insects in each bottle was determined at the beginning of the experiment and two months later. The results recorded in Table 5 show conclusively the effect of the highest level of yeast extract in the growth of No. 103. The lower levels gave results similar to the basal diet without supplements. One cubic centimeter of this alcoholic extract per gram of basal diet (No. 103) stimulated growth to about half adult size in the course of 2 months. This growth was not maximum compared to that obtained with the stock diet of wheat and milk, but it showed the stimulating effects of the extract quite clearly.

Two weeks after this experiment with casein was started, another was begun using gelatin instead of protein in the basal diet.

The same number of groups was employed for each diet except that ten groups of ten each were used for No. 108. The mean weights for these are recorded at the end of

2 and 3 months. Here again there is a marked stimulus to growth in the case of those allowed a supplement of 1 cc. of alcoholic extract per gram of basal diet (No. 107). There is little difference at the end of 2 and 3 months, indicating the shorter period was adequate for preliminary testing of extracts.

TABLE 5

THE EFFECT OF SUPPLEMENTING CASEIN AND GELATIN DIETS WITH DIFFERENT LEVELS OF ALCOHOLIC EXTRACT OF YEAST AND EXTRACT WITH MUCH OF THE NITROGEN REMOVED

Diet No.	Supp. in Cc. per 5 G. Feed	Mean Wt. Start (Mg.)	Mean Wt. 2 Mo. (Mg.)	Mean Wt. 3 Mo. (Mg.)
	"Casein" EtOH Exts. Yeast			
104	0.0	2.5	7.6	....
101	0.1	3.5	8.3	....
102	1.0	3.4	9.2	....
103	5.0	3.3	24.4	....
	'Gelatin" EtOH Exts. Yeast			
108	0.0	1.9	5.5	7.3
105	0.1	2.3	5.9	6.2
106	1.0	2.2	8.2	9.0
107	5.0	2.2	16.5	18.2
	"Casein" Pb (AcO)$_2$Na$_2$CO$_3$ Pptd. Exts. Yeast			
104*a*	0.0	4.9	10.2	14.2
110	1.0	4.6	10.1	18.6
111	5.0	4.6	11.9	20.4
112	20.0	5.0	18.0	35.1
	"Casein" EtOH Ext. Yeast			
103*a*	5.0	5.4	12.0	46.7

The analysis of the yeast extract used above gave the following values per cc.:

N = 0.039 mg.
P = 0.009 mg.
Dry matter = 9.6 mg.
Amino N = 0.1–0.2 mg. (higher value after 30 mins. in the Van Slyke App.)

To remove part of this nitrogen, 300 cc. of the alcoholic extract was treated with 3 cc. of saturated lead acetate followed by 3 cc. of 10 per cent sodium carbonate. This was filtered, centrifuged, and then treated with H_2S. After filtration it was made slightly acid with HCl and again saturated with H_2S. The filtrate from this procedure was reduced in volume on the steam bath, neutralized with NaOH, and finally made to a volume of 300 cc. with EtOH.

About one-fourth of the original nitrogen was still left in the solution while about three-fourths was removed by this precipitation.

To test the filtrate after treatment with mercuric acetate and sodium carbonate, three levels of the filtrate were fed as supplements to the casein basal diet used in 104. One, five, and twenty cubic centimeters of the filtrate were dried on 5-gram samples of basal diet (Nos. 110, 111, and 112). For comparison Diets 104*a* and 103*a* were used. The former contained no supplement and the latter 5 cc. of the original alcoholic extract. These diets with extract supplements are termed Nos. 110, 111, and 112.

These insects were weighed at monthly intervals for 3 months. The mean weights and the number alive are given in Table 5. These results indicate that the filtrate from the mercuric-acetate–treated solution was still potent in stimulating growth but much less effective than the original alcoholic extract of yeast. The development of mature adults upon the diet containing the yeast extract (103*a*) in contrast to that containing none (104*a*) was evident at the time of the last weighing.

The removal of much of the nitrogen from the alcoholic extract may prove useful in future work in testing the need for essential amino acids.

COMPARISON OF DIFFERENT LEVELS OF B_1 FED TO INDIVIDUALS AND TO GROUPS

In order to test a potent source of B_1 at various levels and at the same time to determine if groups instead of individuals can be used for such assays, the following experiments were run.

The same basal diet with casein as a protein source as that used previously was supplemented with B_1 concentrate from rice polishings (Burroughs, Welcome and Company). It was included in the diet at levels of 0, 0.05, 0.5, and 5 per cent. A positive control fed the stock diet was included in the series. Three bottles of ten individuals each were used for each diet as well as ten insects in individual cages. These were weighed at the beginning, the tenth day, and one month. Table 6 gives a summary of results.

These data indicate that there is little advantage in using individuals in preference to groups at the present state of testing. The conclusions would have been the same in either case.

No specific response to B_1 concentrate was obtained whatever the level. The addition of B_1 to the basal diet makes little improvement and the growth rate does not compare favorably between synthetic diets and natural stock diet. The basal diet is apparently highly deficient in factors other than B_1.

THE ETHER-SOLUBLE PORTION OF THE ALCOHOLIC EXTRACT

Four hundred cubic centimeters of the alcoholic extract were evaporated to a syrup. This was extracted with ethyl ether. A fine white powder and an orange paste remained behind. The white powder dissolved readily in EtOH but the syrup only dissolved after considerable boiling.

For this test insects from Diet 108 that had grown little were selected. It was assumed that these would resume growth if the extracts were potent and fail if they were not.

Neither the alcohol nor the ether-soluble fractions stimulated growth in the course of 65 days. Either the extracts were not potent or the insects could not respond after their period of retardation upon Diet 108.

THE FEEDING OF CONCENTRATES OF B_1 AND G

A basal diet was employed consisting of casein 40, salt mixture 4, and starch 66. To each 100 grams of this mixture were added 3 grams of cottonseed oil. Experiments were run in triplicate using bottles with ten insects in each.

TABLE 6

Diet		Mean Wt. 10 Days	Mean Wt. 1 Month	No. Alive 10 Days	No. Alive 1 Month
Basal alone......	Group	3.6	5.0	10	8
	Group	4.2	5.0	10	9
	Group	3.8	5.0	10	9
	Individual	4.1	4.1	10	6
Basal plus 0.05....	Group	3.8	8.0	7	6
	Group	5.1	7.0	8	7
	Group	4.3	5.0	10	8
	Individual	4.5	3.8	10	7
Basal plus 0.5.....	Group	5.1	7.0	10	8
	Group	4.3	6.0	10	8
	Group	4.3	7.0	10	7
	Individual	4.9	5.2	10	7
Basal plus 5.0.....	Group	6.2	9.0	9	5
	Group	5.5	8.0	10	3
	Group	5.1	8.0	8	4
	Individual	6.0	5.2	10	7
Stock diet.........	Group	14.6	35.0	10	9
	Group	11.2	42.7	10	10
	Individual	19.0	57.5	10	10

The purpose of the first series was to add B_1 and G at two different levels and to add each separately. The sources of B_1 and G were a rice polish extract of the Burroughs, Welcome Company and "XXX liquor" of the Borden Company. Groups in these trials were weighed 10 and 20 days after the beginning as well as at the start. The results of these experiments are summarized briefly in Table 7.

These data indicate clearly that a basal diet supplemented with sources of both B_1 and G at relatively high levels gives relatively poor growth compared to a mixture of wheat and milk. This would seem to indicate that the basal diet is not satisfactory or that other vitamin fractions are essential. The best results upon the synthetic diets were obtained with a mixture of B_1 and G at relatively high levels, but these differed too little from the poorest and were too far from those fed the natural foods to be of much significance. This experiment was repeated with higher levels of the concentrate, but the results were the same and little growth resulted.

The effect of autoclaving the stock diet was now tried. The mixture of wheat flour and dry skim milk was autoclaved dry after making it into a mush. The value of the stock diet did not seem impaired by this treatment which was made under the same conditions as those used for preparing yeast for B_1 assays.

FURTHER STUDIES WITH B FRACTIONS[1]

In order to test other B fractions a new series was now run. The composition of the diets and the fractions used as well as the results are summarized in Table 8. The G was the impure flavine from yeast (Kinnersley, O'Brien, Peters, and Reader, 1933). The "basal" diet was the mixture used at Oxford for assaying B fractions with rats. These results indicate further that yeast contains other fractions essential for this species.

TABLE 7

FAILURE OF B_1 AND G SUPPLEMENTS TO PERMIT GROWTH

Diet	Mean Wt. Begin. (Mg.)	Mean Wt. 10 Days (Mg.)	Mean Wt. 20 Days (Mg.)
Basal+0.1 per cent "B_1"+0.1 per cent "G"	1.7	2.9	3.8
Basal+0.1 per cent "G"	1.8	2.5	3.2
Basal+1.0 per cent "B_1"+1.0 per cent "G"	1.8	2.7	4.3
Basal+1 per cent "B_1"	1.7	2.9	3.5
Basal+3 per cent "G"	1 6	2.6	3.4
Wheat-milk (stock)	1.7	8.4	23.1
Basal alone	....	2.6	3.0

TABLE 8

GROWTH WITH B_1 AND G_2 SUPPLEMENTS

Diet	Wt. Start	Wt. 20 Days	No. Alive 20 Days
Basal alone	1.06	3.1	5
Basal+B_1+G	1.06	4.9	1
Basal+B_1	1.06	3.5	7
Basal+G	1.06	1.7	2
Stock diet	1.06	9.0	8

SUPPLEMENTING BASAL DIETS WITH YEAST, WATER EXTRACT OF YEAST, AND YEAST RESIDUE AFTER WATER EXTRACTION

As an additional advance, comparisons were made between fresh yeast, its water extract, and the residue after water extraction. One hundred and fourteen grams of fresh yeast were extracted with small portions of boiling water. This was repeated six times and centrifuged between each. This yielded a total of about 60 cc. of extract. In our table of diets 5 per cent in the case of the extracts means that 5 cc. of this liquid was mixed with 100 grams of the basal diet. Duplicate groups of 14 each and of almost the same mean weight at the start were used. The diets and results are summarized in Table 9.

---

[1] These studies were made in the biochemistry laboratory of Oxford University. The author wishes to express his appreciation to Professor R. Peters and to Dr. J. R. O'Brien for supplying B fractions and providing facilities for these studies.

TABLE 9

EFFECT OF FEEDING YEAST AND AQUEOUS EXTRACTS

Diet	Wt. after 4 weeks (mg.)	Diet	Wt. after 4 weeks (mg.
Basal+10 per cent yeast (fresh)	15.6	Basal+10 per cent extract	5.8
	13.4		7.5
Basal+20 per cent yeast (fresh)	11.2	Basal+20 per cent extract	4.1
	16.6		8.6
Basal+10 per cent residue	8.6	Basal alone	2.0
	4.9		4.4
Basal+20 per cent residue	6.8	Stock diet	17.4
	4.2		13.2

These results indicated that the basal diet supplemented with fresh yeast was almost equal if not equal to the stock diet. The water extracts of yeast and the yeast residue yielded intermediate results between the basal diet alone and the yeast diets. As a whole both the residue and the extract proved poor compared to the yeast and the stock diet.

FAT SOLUBLE VITAMINS

To determine the effect of extracting the stock diet with fat solvents four experiments were run. Three groups of ten each were fed each diet:

Diet A.—Wheat-milk stock diet extracted with ethyl ether—residue only
Diet B.—Wheat-milk stock diet extracted with ethyl ether and the extract recombined with the diet
Diet C.—The same as A except petroleum ether was used
Diet D.—Diet C was recombined with the extract

Table 10 shows the weight increases for each of the groups.

TABLE 10

GROWTH UPON STOCK DIET OF WHEAT AND MILK EXTRACTED WITH ETHYL AND PETROLEUM ETHER

Diet	10 Days Mean Wt. (Mg.)	20 Days Mean Wt. (Mg.)
A. Wheat-milk-residue after extraction with Et_2O	9.4	19.7
B. Wheat-milk extracted with Et_2O and recombined with extract	10.9	29.0
C. Wheat-milk extracted with petroleum ether	8.0	18.0
D. Wheat-milk extracted with petroleum ether and recombined	10.9	30.5

The diets with the residues and the extracted materials returned were superior to the residues alone. Either some essential had been removed by the ethers or the physical condition of the diet had become unfavorable. If alcohol had preceded the ether extractions, more marked differences might have resulted. It is unlikely that either vitamin A or D is responsible for the difference although this must ultimately be checked by direct test. E may be important. Specific fatty acids and cholesterol may be essential.

ALCOHOLIC-ETHER EXTRACTION OF FOODSTUFF

In another experiment the stock diet was extracted with ethyl alcohol (95 per cent) for 48 hours. This was then followed by extraction with petroleum ether for the same time. These resulting fractions were tested in triplicate.

These data which are not included indicated that extraction with alcohol and ether removes something that is essential and which is not made good by vitamins A, D, B_1, and G. The better growth upon the stock diet indicated that the extraction and recombination may have actually destroyed some essential substances. The other possibility is that the solvents left a toxic residue

TABLE 11

LIFE SPAN OF *Blattela germanica*

Source of Insects	Diet Fed for the Remaining Life	Mean Wt. Attained in Mg.	Wt. Extremes in Mg.	Mean Life-Span in Days	Life-Span Extremes in Days
Diet 105........	"Gelatin" basal+2 cc. Ext. (yeast) per 5 grams	6.0	4.2– 9 5	158	107–245
Diet 107........	"Gelatin" basal+20 cc. Ext. (yeast) per 5 grams	22.2	10.4– 35.2	206	107–317
Diet 103........	"Casein" basal+5 cc. Ext. (yeast) per 5 grams	56	21 –106	179	78–314
Diets 101, 102, 104	Casein basal+2 cc. Ext. (yeast) per 5 grams	16.4	6.4– 39.3	175	133–263

LIFE SPAN DATA

This species seems to be especially adapted for population and life span studies. It does not pass its life-cycle in the midst of its food and therefore fewer complications are introduced from excreta and bacterial action. These factors are of interest in interpreting data from insects and in drawing conclusions to assist in problems with higher animals. Individuals from the preceding assays were available for life-span studies after failure to grow owing to vitamin deficiencies.

In the present study an attempt was made to measure the life-span of individuals retarded in growth and never allowed to mature in comparison with those reared to maturity. In each case the retardation was effected by limiting the vitamin B allowed in the alcoholic extract of yeast. The individuals studied were employed originally for the assays described previously. Pertinent data regarding life-span are shown in Table 11.

These data indicate that the normal life-span for this species held at 25° C is seven months or less. These data also show that growth can be retarded for five months. In early experiments growth was resumed after retardation but no studies have been made after retardation of as large a fraction of the total life-span as five months.

SUMMARY

Methods for employing the ordinary cockroach, *germanica*, in nutrition studies have been devised. The conversion of matter into body tissue by this species indicates that

it is nearly three times as efficient as the better domestic animals such as swine. An extensive series of tests to determine the vitamin needs of this insect indicates that it must have certain essential factors found in yeast. These factors seem to be partly soluble in water and partly in lipid solvents such as alcohol and ether. These factors seem to be different from B_1 and flavine. No evidence indicates any need for fat-soluble vitamins A and D. Life-span data for this species indicate that all die within a year. Individuals whose growth has been checked by vitamin deficiencies can live for as much as five months.[2]

LITERATURE CITED

ARMSBY, H. P., and MOULTON, C. R. 1925. The animal as a converter of matter. New York.

BARRETT, J. F., and JONES, E. B. 1932. A colorimetric method for the direct determination of urea in urine. Biochem. Jour., 26, 1246.

CROWELL, M. F., and McCAY, C. M. 1937. Nutritional studies of the webbing clothes moth. *Tineola bissellielle* Hum. Physiol. Zoöl., 10, 368.

KINNERSLEY, H. W.; O'BRIEN, J. R.; PETERS, R. A.; and READER, VERA. 1933. Large scale preparations of vitamin B_1 and B_4 concentrates. Biochem. Jour., 27, 225.

LUSK, G. 1928. The science of nutrition. Philadelphia.

McCAY, C. M. 1937. The biochemistry of fish. Ann. Rev. Biochem., 6, 445.

———. 1933. An insect test for vitamin B fractions. Proc. Amer. Soc. Biol. Chemists 100, lxviv.

MORRIS, H. P.; NELSON, J. W.; and PALMER, L. S. 1931. The quantitative determination of calcium, magnesium, and phosphorus in feedstuffs and excreta. Jour. Ind. and Eng. Chem., 3, 164.

OSBORNE, T. B., and MENDEL, L. B. 1919. The nutritive value of the wheat kernel and its milling products. Jour. Biol. Chem., 37, 557.

PALMER, L. S., and KENNEDY, C. 1931. The fundamental food requirements for the growth of the rat. Jour. Biol. Chem., 90, 545.

TRAGER, WM. 1935. On the nutritional requirements of mosquito larvae, *Aedes Aegypti*. Amer. Jour. Hyg., 22, 475.

WOLLMAN, E. 1926. Observation sur une lignee aseptique des Blattes (*Blattella germanica*). C. R. Soc. de Biol., 95, 164.

YASUDA, M. 1931. The determination of the iodine number of lipids. Jour. Biol. Chem., 94, 401.

YOUNGBERG, G., and YOUNGBERG, M. 1930. A system of blood phosphorus analysis. Jour. Lab. and Clin. Med., 16, 158.

ZABINSKI, JAN. 1929. The growth of blackbeetles and cockroaches on artificial and on incomplete diets. Jour. Exper. Biol., 6, 360.

---

[2] In the course of these studies the author is indebted for assistance to Dr. Mary Crowell, Dr. J. de Tomasi, and Mr. R. E. Bowers as well as members of the biochemistry department of Oxford University, England.

C. M. Child

CHARLES MANNING CHILD
Professor Emeritus of Zoology
The University of Chicago

This number of *Physiological Zoology* is made up of papers by former students of Professor Child who wrote their Doctor's theses under his supervision Dr Libbie H. Hyman, J William Buchanan, and Lester G. Barth have assisted in the editorial work.

CHARLES MANNING CHILD—VITA

1869	February 2, born Ypsilanti, Michigan, of New England parentage
1890	Ph.B., Wesleyan University, Middletown, Connecticut (Chi Psi Fraternity)
1890–92	Graduate assistant in biology, *ibid.*
1892	S.M., *ibid.*
1894	Ph.D., University of Leipzig, Germany
1894	Naples Zoölogical Station
1894	Assistant in zoölogy, University of Chicago
1895	Associate, *ibid.*
1897	Instructor, *ibid.*
1895–97	Instructor in embryology, Marine Biological Laboratory
1898	Assistant professor of zoology, University of Chicago
1899	Married Lydia Van Meter of Baltimore, Maryland
1902	Naples Zoölogical Station
1909	Associate professor of zoology, University of Chicago
1911	*Die physiologische Isolation von Teilen des Organismus* (Leipzig)
1915	*Senescence and Rejuvenescence* (Chicago)
1915	*Individuality in Organisms* (Chicago)
1916	Professor of zoölogy, University of Chicago
1919	President, American Society of Zoölogists
1921	*The Origin and Development of the Nervous System from a Physiological Point of View* (Chicago)
1924	*Physiological Foundations of Behavior* (New York)
1928	Joined with W. C. Allee to found the journal, *Physiological Zoölogy*, and became first managing editor
1928	Honorary D.Sci., Wesleyan University, Connecticut
1929	Vice-president, Section F, American Association for the Advancement of Science
1930	Visiting professor, Duke University, from February to June
1930–31	Rockefeller Foundation Visiting Professor, Tohoku Imperial University, Sendai, Japan
1932	Chairman, Department of Zoölogy, University of Chicago
1933	Elected to the National Academy of Science
1934	Professor emeritus of zoölogy, *ibid.*
1937	Moved to Palo Alto, California; present address: Jordan Hall, Stanford University, California
1895——	Research worker at the marine biological laboratories at Woods Hole, Friday Harbor, La Jolla, Pacific Grove, and Asamushi (Japan)

Physiological Zoölogy

Vol. XI APRIL 1938 *No. 2*

ANNOTATED BIBLIOGRAPHY OF THE SCIENTIFIC PUBLICATIONS OF PROFESSOR CHARLES MANNING CHILD

LIBBIE H. HYMAN
American Museum of Natural History, New York City
AND
C. D. VAN CLEAVE
Department of Anatomy, University of Pennsylvania

1892*a*. Statistics of "unconscious cerebration." Amer. Jour. Psychol., 5:249–59.

1892*b*. The functions of the nervous system of the Myriapoda. Amer. Nat., 26:1051–55.

In the absence of head or cerebral ganglia, the trunk will, when stimulated, execute normal movements, but weaker and less exact. The effects of partial destruction of the cerebral ganglia are proportional to quantity destroyed; circus movements result when half is destroyed. No difference was found in trunk behavior with respect to level.

1894*a*. Beiträge zur Kenntnis der antennalen Sinnesorgane der Insekten. Zool. Anz., 17:35–38.

1894*b*. Contributions to the knowledge of the antennary sense-organs of insects. Ann. and Mag. of Nat. Hist., 13:372–74.

A translation of 1894*a*. Surveys the occurrence of the antennary sense organ in the orders of insects.

1894*c*. Ein bisher wenig beachtetes antennales Sinnesorgan der Insekten mit besonderer Berücksichtigung der Culiciden und Chironomiden. Zeitschr. f. wiss. Zool., 58:475–528.

This, Professor Child's thesis at the University of Leipzig, has become a standard reference in entomological works. A detailed account of the histology and innervation of an antennary sense-organ, especially in the lower Diptera; perception of vibrations is suggested as the function.

1896. Translation of E. Haase: Researches on mimicry on the basis of a natural classification of the Papilionidae. Part II. Stuttgart and London. Pp. 154; 8 plates.

1897*a*. Centrosome and sphere in the ovarian stroma of mammals. Abstract, Zoölogy Club, University of Chicago, meeting of January 6, 1897. Science, N.S., 5:231–32.

1897*d*. Centrosome and sphere in cells of the ovarian stroma of mammals. A preliminary communication. Zoöl. Bull., 1:87–94.

Stroma cells of the pregnant ovary of rabbit and dog contain spheres and centrioles, although they do not divide.

1897*b*. I. Cleavage of the egg of *Arenicola*. II. The oblique cleavage and its relation to the mosaic theory. Abstract, Zoölogical Club, University of Chicago. Science, N.S., 5:629–31.
1897*c*. A preliminary account of the cleavage of *Arenicola cristata*, with remarks on the mosaic theory. Zoöl. Bull., 1:71–85.
1898*a*. Some features of the oögenesis of *Sternaspis*. Science, N.S., 7:104–5.
1898*b*. The maturation and fertilization of the egg of *Arenicola marina*. Trans. N.Y. Acad. Sci., 16:387–94.
1898*c*. The axes of the annelid egg. Science, N.S., 8:140.
1900*c*. The significance of the spiral type of cleavage and its relation to the process of differentiation. Biol. Lectures, Marine Biol. Lab. Woods Hole, for 1899, pp. 231–65.
1900*d*. The early development of *Arenicola* and *Sternaspis*. Arch. f. Entw'mech., 9:587–723.

Final extensive paper to which the preceding six are preliminary; a classic on cell lineage. Spiral cleavage is interpreted as a form of cleavage offering the least physical obstacle to intercommunication of the components. Bilateral cleavage is later superimposed on the spiral type and leads directly to the formation of the larva; hence called "morphogenetic cleavage." In annelids and mollusks morphogenetic cleavage is increased at the expense of spiral cleavage. The cleavage pattern of different species is identical only as long as the spiral type obtains. The differentiation of larval organs is related to their functional necessity, and hence may occur at different times in the development of different species. Bilateral cleavage is interpreted as accomplishing a symmetrical distribution of materials to the larva. The mosaic theory is rejected on the grounds of the failure of exact cell homology in many cases, the differences in size and structure of blastomeres having the same fate, and the ability of portions of determinate embryos to regulate to at least some extent. A strictly cellular theory of development must always be inadequate; the explanation of development must be sought in the organization of the egg and of each stage as a whole. Hertwig's view that the fate of a cell is a function of its position is accepted.

1900*a*. The egg of the *Stichostemma*. Science, N.S., 11:249–50.
1901*c*. The habits and natural history of *Stichostemma*. Amer. Nat., 35:975–1006.

The external features, occurrence, movements, reactions, food, and enemies of the fresh-water nemertean are described. The habit of forming a slime coat may have protective value. Self-fertilization is probably the rule. Each ovary is a sac with a syncytial wall; this wall gradually bulges into the cavity as a single egg, which finally constricts off and flows through the small pore to the exterior.

1900*b*. A specimen of *Nais* with a bifurcated prostomium. Anat. Anz., 17:311–12.
1900*e*. Abnormalities in cestodes. Abstract, Zoölogical Club, University of Chicago. Science, N.S., 12:28–29.
1900*f*. Abnormalities in the cestode *Moniezia expansa*. I. Imperfect and partial proglottids. Biol. Bull., 1:215–50.

1900*g*. Abnormalities in the cestode *Moniezia expansa*. II. 1. Spiral abnormalities. 2. Other abnormalities. Biol. Bull., 1:261–90.

1902*b*. Abnormalities in the cestode *Moniezia expansa*. III. Biol. Bull., 3:95–114, 143–60.

The abnormalities consist chiefly of imperfect separation of proglottids. Study of the development of the proglottids and their organs showed that the external abnormalities are but a surface expression of internal developmental anomalies.

1901*a*. Regulation in *Stenostomum*. Abstract, Zoölogical Club, University of Chicago. Science, N.S., 14:28–29.

1901*b*. Fission and regulation in *Stenostomum leucops*. Abstract, Meeting of American Morphological Society, Baltimore, December, 1900. Biol. Bull., 2:329–31.

1902*a*. An experimental study of regulation in *Stenostoma*. Science, N.S., 15:527–28.

1902*c*. Studies on regulation. I. Fission and regulation in *Stenostoma*. Part I. The structure and movements of *Stenostoma* and the process of fission. Part II. The theory of mechanical regulation. Arch. f. Entw'mech., 15:187–237.

1902*d*. Studies on regulation. I. Fission and regulation in *Stenostoma*. Part III. The regulation of zooids and groups of zooids. Arch. f. Entw'mech., 15:355–420.

1903*b*. Studies on regulation. II. Experimental control of form-regulation in zooids and pieces of *Stenostoma*. Arch. f. Entw'mech., 15:603–37.

1903*e*. Studies on regulation. III. Regulative destruction of zooids and parts of zooids of *Stenostoma*. Arch. f. Entw'mech., 17:1–40.

These papers on *Stenostomum* are the first of a long series of researches on the role of mechanical factors, especially tensions resulting from locomotion and attachment, in form-regulation. *Stenostomum* attaches by the tail and ventral surface; such tensions induce a conoidal form. Regulation can be controlled by preventing attachment through keeping the pieces moving; this inhibits or delays the formation of a tail. Typical increase in length, decrease in breadth, of regenerating pieces result from tail attachment and are greatest in pieces with heads, since these attach. Pieces without heads do not attach, and change shape slowly. *Stenostomum* has no "normal" form; its form is the result of its activities; morphallaxis is brought about by differences in tensions. In pieces containing parts of more than one zooid, the oldest zooid absorbs the younger zooids or portions thereof anterior to it.

1903*a*. With A. N. YOUNG. Regeneration of the appendages in nymphs of the Agrionidae. Arch. f. Entw'mech., 15:543–602.

Emphasis is placed on the correlation between the regeneration of muscles and tendons and the regeneration of legs and formation of joints. Normal segmentation of the leg into joints depends on regeneration of normal muscle arrangements; the tarsal claws do not become articulated unless tendons reach them.

1903*f*. Form-regulation in Coelentera and Turbellaria. Smithsonian Misc. Coll. 45:134–43.

Summary of work on the regeneration of *Leptoplana*, *Cestoplana*, *Cerianthus*, and *Tubularia*.

1903*c*. Form-regulation in *Cerianthus*. I. The typical course of regeneration. Biol. Bull., 5:239–60.

1903*d*. Form-regulation in *Cerianthus*. II. The effect of position, size, and other factors upon regeneration. Biol. Bull., 5:304–19. II. Continued. Biol. Bull., 6:1–11.

1904*a*. Form-regulation in *Cerianthus*. III. The initiation of regeneration. Biol. Bull., 6:55–74.

1904*b*. Form-regulation in *Cerianthus*. IV. The role of water-pressure in regeneration. Biol. Bull., 6:266–86.

1904*d*. Form-regulation in *Cerianthus*. V. The role of water-pressure in regeneration: further experiments. Biol. Bull., 7:127–53.

1904*c* Form regulation in *Cerianthus*. VI. Certain special cases of regulation and their relation to internal pressure. Biol. Bull., 7:193–214.

1904*f*. Form regulation in *Cerianthus*. VII. Tentacle-reduction and other experiments. Biol. Bull., 7:263–79.

1905*a*. Form regulation in *Cerianthus*. VIII. Supplementary partial discs and heteromorphic tentacles. Biol. Bull., 8:93–122.

1905*b*. Form regulation in *Cerianthus*. IX. Regulation, form and proportion. Biol. Bull. 8:271–89.

1908*b*. Form regulation in *Cerianthus aestuarii*. Biol. Bull., 15:27–53.

Cerianthus pieces collapse; the ends close; and the piece distends through water intake. Such distension is a necessary condition for normal regeneration. If prevented by keeping the aboral end open, regeneration is delayed or inhibited, and regenerated tentacles are short and stumpy. The direct factors are probably water currents striking against the anterior end of the piece. Regeneration is greatest in rate and amount orally and decreases basally. In oblique pieces the most anterior angle regenerates first. Regeneration is not proportional to the size of the piece but is relatively greater the smaller the piece. The change of form of pieces in the direction of normal elongated shape occurs chiefly after the regeneration of the oral end.

1904*c*. Studies on regulation IV. Some experimental modifications of form-regulation in *Leptoplana*. Jour Exper. Zoöl., 1:95–133.

1904*g*. Studies on regulation. V. The relation between the central nervous system and regeneration in *Leptoplana:* posterior regeneration. Jour. Exper. Zoöl., 1:463–512.

1904*i*. Studies on regulation. VI. The relation between the central nervous system and regeneration in *Leptoplana:* anterior and lateral regeneration. Jour. Exper. Zoöl., 1:513–57.

1905*c*. Studies on regulation. VII. Further experiments on form-regulation in *Leptoplana*. Jour. Exper. Zoöl., 2:253–85.

Further pursuit of the idea of the importance of pressure and tension in regeneration. In curved pieces of the polyclad *Leptoplana* the regenerated tail curves in the direction of the principal tension. Regions develop in a typical form because they function in a characteristic manner. The regenerated tail is shorter and less pointed the less active the piece. The amount and completeness of anterior and lateral regeneration are also correlated with the characteristic motor activity. Changes in proportions are the result of tensions during locomotion.

1905*d*. Studies on regulation. VIII. Functional regulation and regeneration in *Cestoplana*. Arch. f. Entw'mech., 19:261–94.

1905*f*. Studies on regulation. IX. The positions and proportions of parts during regulation in *Cestoplana* in the presence of the cephalic ganglia. Arch. f. Entw'mech., 20:48–75.

1905*g*. Studies on regulation. X. The positions and proportions of parts during regulation in *Cestoplana* in the absence of the cephalic ganglia. Arch f. Entw'mech., 20:157–86.

1907*o*. Studies on regulation. XI. Functional regulation in the intestine of *Cestoplana*. Jour. Exper. Zoöl., 4:357–98.

1910*e*. The central nervous system as a factor in the regeneration of polyclad Turbellaria. Biol. Bull., 16:277–96.

Pieces with ganglia elongate and become tapering, owing to locomotor activity. Pieces without ganglia regenerate but little; they undergo redifferentiation of the digestive branches in relation to mechanical conditions. In headless pieces the pharynx is more anterior the more posterior the level; pieces behind the old pharynx do not form a new pharynx. In starvation the intestine degenerates from periphery to center and from the ends toward the pharynx. In regeneration, branches of the intestine degenerate and new ones arise, corresponding in arrangement to the appropriate body region. In headless pieces without pharynx the intestine regenerates. Degeneration of old intestinal branches and re-formation of new ones are assigned to mechanical conditions.

1905*e*. Regeneration in nudibranchs. Science, N.S., 21:851.

1906*a*. Contributions toward a theory of regulation. I. The significance of the different methods of regulation in Turbellaria. Arch. f. Entw'mech., 20:380–426.

1906*e*. The relation between functional regulation and form-regulation. Jour. Exper. Zoöl., 3:559–82.

1907*d*. The localization of different methods of form-regulation in *Polychoerus caudatus* Arch. f. Entw'mech., 23:227–48.

These four papers continue the thesis that regulation results from the regenerating part acting functionally like the part removed. In different forms and different levels of the same form, redifferentiation (morphallaxis) and regeneration proper occur to different degrees. The more the region where a part was removed is able to substitute functionally for the part removed, the greater the role of redifferentiation of old tissue in the regulation; and regeneration occurs when functional substitution is limited to the cut surface and immediately adjacent tissue. Regulation is defined as a return or approach to a physiological or functional equilibrium after such equilibrium has been disturbed.

1906*c*. Some considerations regarding so-called "formative substances." Biol. Bull., 11:165–81.

Proponents of formative substance theories have failed to understand that, since organic structure is essentially a space arrangement of materials, formative substances would have to act by determining such an arrangement. A gradation of head-forming or tail-forming substances would have in itself to be explained; further head and tail are highly complex parts. Formative substance theories err not only in these points but in failing to recognize that all processes are part of the functional activity of the organism.

1907*j*. Some corrections and criticisms. Arch. f. Entw'mech., 24:131–46.

1907*f*. An analysis of form-regulation in *Tubularia*. I. Stolon-formation and polarity. Arch. f. Entw'mech., 23:396–414.

1907*g*. An analysis of form-regulation in *Tubularia*. II. Differences in proportion in the primordia. Arch. f. Entw'mech., 23:415–44.

1907*h*. An analysis of form-regulation in *Tubularia*. III. Regional and polar differences

in the relation between primordium and hydranth. Arch. f. Entw'mech., 23: 445–56.

1907*i*. An analysis of form-regulation in *Tubularia*. IV. Regional and polar differences in the time of hydranth-formation as a special case of regulation in a complex system. Arch. f. Entw'mech., 24:1–28.

1907*n*. An analysis of form-regulation in *Tubularia*. V. Regulation in short pieces. Arch. f. Entw'mech., 24:285–316.

1907*o*. An analysis of form-regulation in *Tubularia*. VI. The significance of certain modifications of regulation: polarity and form-regulation in general. Arch. f. Entw'mech., 24:317–49.

1909*b*. The regulation of mutilated primordia in *Tubularia*. Arch. f. Entw'mech., 27: 106–13.

In *Tubularia* pieces the tendency to form stolons aborally is greater the more proximal the piece. The proportions of the parts of the primordium of the hydranth vary with level of piece. Hydranths form earlier on distal than on proximal pieces, and on longer than on shorter pieces; aboral hydranths arise later than oral on a piece. The shorter the piece, the longer the hydranth in proportion, until very short pieces become all hydranth with no stalk. Very short pieces produce simple or double complete or incomplete hydranths. The formation of an aboral hydranth is a case of asexual reproduction. If the physiological difference between the two ends of a piece is sufficiently great, a single structure of reduced size results; if the difference is slight, bipolar structures result. The polarity of *Tubularia* is not fixed but reacts to, and is alterable by conditions.

1906*b*. The relation between regulation and fission in *Planaria*. Biol. Bull., 11:113–23.

1908*a*. Physiological basis of restitution of lost parts. Jour. Exper. Zoöl., 5:485–502. Autoreferat in Arch. f. Entw'mech., 26:501.

1909*b*. The regulatory change of shape in *Planaria dorotocephala*. Biol. Bull., 16:277–96.

Application of the theory of the role of function in regulation to *Planaria;* a part can be replaced only when some remaining part is physiologically sufficiently similar to it to perform its functions at least qualitatively. The position of the pharynx in regenerating pieces depends on how much such pieces react like heads or tails, respectively. In dilute anesthetics, which prevent locomotion but permit regeneration, the normal change of shape of pieces does not occur; such change of shape is dependent on locomotion. The presence of a posterior zooid in *Planaria* is indicated for the first time.

1908*c*. Driesch's harmonic equipotential systems in form-regulation. Biol. Centralbl., 28:577–88, 609–23.

A postulate of Driesch's system is retention of proportionality with every reduction. Proportionality is not retained with reduction of size of *Tubularia* pieces but varies with level and size. The postulate that in organisms, in contrast with machines, each part can do everything is also not in accord with the facts of regeneration.

1908*d*. Regulation of *Harenactis attenuata* in altered environment. Biol. Bull., 16:1–17.

1909*a*. Experimental control of certain regulatory processes in *Harenactis attenuata*. Biol. Bull., 16:47–53.

1909*c*. Factors of form regulation in *Harenactis attenuata*. I. Wound reaction and restitution in general and the regional factors in oral restitution. Jour. Exper. Zoöl., 6:471–506. Autoreferat in Arch. f. Entw'mech., 28:440.

1909*d*. Factors of form regulation in *Harenactis attenuata*. II. Aboral restitution, heteromorphosis and polarity. Jour. Exper. Zoöl., 7:65–96.

1909*e*. Factors of form regulation in *Harenactis attenuata*. III. Regulation in "rings." Jour. Exper. Zoöl., 7:353–95. Autoreferat in Arch. f. Entw'mech., 29:198–99.

1910*f*. Further experiments on adventitious reproduction and polarity in *Harenactis*. Biol. Bull., 20:1–13.

On removal from its sand burrow the slender *Harenactis* slowly shortens to a typical anemone form with expanded oral and pedal disks; external conditions therefore determine the shape. The results of a cut into the side of the upper end depend on internal distension; without distension, a part degenerates. Level of piece determines rate of closure, amount of restitution, number of tentacles, etc. Pieces through the esophagus usually regenerate biaxial oral ends; below the esophagus no aboral head arises. The regeneration of the foot is greatest aborally and decreases distally. Regional differences are both qualitative and quantitative. Rings result from the union of the two cut surfaces into a doughnut shape if mesenteries and esophagus are removed. Such rings regenerate irregular tentacles or groups of tentacles along or near the line of union. The old polarity is lost, and tentacle groups represent adventitious new polarities.

1904*h*. Amitosis in *Moniezia*. Anat. Anz., 25:545–58.

1906*d*. The development of germ cells from differentiated somatic cells in *Moniezia*. Anat. Anz., 29:592–97.

1907*a*. Studies on the relation between amitosis and mitosis. I. Development of the ovaries and oögenesis in *Moniezia*. Biol. Bull., 12:89–114.

1907*b*. Studies, etc. II. Development of the testis and spermatogenesis in *Moniezia*. Biol. Bull., 12:175–90.

1907*c*. Studies, etc. II. Continued. Biol. Bull., 12:191–224.

1907*e*. Amitosis as a factor in normal and regulatory growth. Anat. Anz., 30:271–97.

1907*k*. Studies, etc. III. Maturation, fertilization, and cleavage in *Moniezia*. Biol. Bull., 13:138–60.

1907*l*. Amitosis and mitosis in normal and regulatory growth. Proc. Seventh Intl. Zoöl. Congress, pp. 344–46.

1907*p*. Studies, etc. IV. Nuclear division in the somatic structures of the proglottids of *Moniezia*. V. General discussion and conclusions concerning amitosis and mitosis in *Moniezia*. Biol. Bull., 13:165–84.

1910*a*. The occurrence of amitosis in *Moniezia*. Biol. Bull., 18:109–19.

1911*e*. The method of cell division in *Moniezia*. Biol. Bull., 21:280–96.

Amitosis was found to be the characteristic mode of division in rapidly growing regions, in the formation of the gonads and their ducts, nephridia, nerve cords, and interproglottidal glands. Testes may originate from muscle cells which divide amitotically to become a syncytial mass; the spermatogenic divisions are mitotic. The ovaries arise from parenchymal cells by amitotic divisions; maturation and early cleavages are mitotic, but later cleavages are amitotic. Amitosis is believed to be characteristic of rapidly growing and highly active regions, as in developing embryos and regenerating pieces of hydroids, planarians, etc.

1907*m*. Correlation in regulation. Proc. Seventh Intl. Zoöl. Congress, pp. 497–500.

1910*d*. Physiological isolation of parts and fission in *Planaria*. Arch. f. Entw'mech. (Festschrift für Roux, Vol. II), 30:159–205.

1911*a*. Die physiologische Isolation von Teilen des Organismus als Auslösungsfaktor der

Bildung neuer Lebewesen und der Restitution. Vorträge und Aufsätze über Entwicklungsmechanik (ed. by W. Roux), 11. Pp. 157.

The problem of the unity of the organism is the problem of correlation. The chief factor in correlation is antero-posterior dominance: each level dominates the region behind and is dominated by that in front. The domination of the anterior end over posterior regions declines with distance; hence each individual can reach only a limited size; beyond that size there occurs a physiological isolation, essentially similar to a physical isolation. Besides this distance factor, the length of the individual and the range of dominance of the head are dependent on the degree of activity of the dominant region, the efficiency of conduction, and the degree of receptivity of the posterior regions. The physiological isolation of posterior parts can be experimentally altered by way of these four factors. The results of the physiological isolation of parts depends on the capacities of these parts. If they are incapable of regeneration, physiological isolation results in atrophy, degeneration, absorption, or death of the parts in question. Physiological isolation probably accounts for the absorption or casting off of embryonic organs during metamorphosis; also for the self-differentiation of embryonic regions. If parts physiologically isolated are capable of regeneration, then asexual reproduction occurs. The distance factor is plainly evident in the formation of zooids in naids, planarians, and *Stenostomum*, in the budding of hydroids, etc. Physiological isolation may permit the transformation of parts under the action of external factors, as the change from vegetative to reproductive organs in plants. The localization of sex cells may be the result of physiological isolation. Fragmentation, adventitious budding, colony formation—all involve the principle of physiological isolation. Neoplasms may also be explained as an escape from physiological control.

In planarians increase in length results in the formation of zooids in the postpharyngeal region and consequent fission. Fission can be induced at a length much shorter than normal by starvation, decapitation, dilute anesthetics, rise of temperature, and can be delayed beyond the normal length by low temperature.

1910*c*. The regulatory processes in organisms. Jour. Morph., 22:171–222.

The existence and continuity of organic individuality are dependent on physiological correlation. Correlative factors classify as mass, or mechanical; transportative; and transmissive, or dynamic. Continued existence is continued equilibration; regulation is the return to a state of dynamic equilibrium after the previous state has been disturbed, and embraces a wide range of phenomena, from a reflex act to regeneration. Life is a succession of equilibrations. The organism is compared to a river, whose structure is the result of past functioning but also influences present function. The form and shape of the river and its banks are the result of the activity of the stream; so, also, the organism is constructed by functioning, not for function. The regulatory processes classify as quantitative or compensatory and qualitative or transformative. In the regeneration of a piece there is a transformation of the whole piece, a reconstitution; it results from the attempt of the piece to function like the whole.

1911*b*. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. I. The axial gradient in *Planaria dorotocephala* as a limiting factor in regulation. Jour. Exper. Zoöl., 10:265–320.

1911*d*. Experimental control of morphogenesis in the regulation of *Planaria*. Biol. Bull. 20:309–31.

1911*f*. Studies on the dynamics of morphogenesis, etc. II. Physiological dominance of anterior over posterior regions in the regulation of *Planaria dorotocephala*. Jour. Exper. Zoöl., 11:187–220.

1911*g*. Studies on the dynamics of morphogenesis, etc. III. The formation of new zooids in *Planaria* and other forms. Jour. Exper. Zoöl., 11:221–80.

1911*h*. With E. V. McKie. The central nervous system in teratophthalmic and teratomorphic forms of *Planaria dorotocephala*. Biol. Bull., 22:39–60.

1912*b*. Antero-posterior dominance in *Planaria*. Abstract. Science, N.S., 35:935–36.

The kind and amount of regeneration vary with level and size of piece. Grades of head varying from normal through inhibited types to headlessness occur. The rate of head formation, the frequency of normal heads and eyes, head size, and the distance of the pharynx from the head decrease along the anteroposterior axis, facts indicating the existence of an anteroposterior physiological gradient (first mentioned in 1911*b*). An increase in these matters in postpharyngeal regions shows the presence there of one or more zooids physiologically. The inability of pieces to form structures anterior to their own level unless some grade of head is first regenerated evidences head-dominance. The regulation of a piece to a whole consists essentially of the formation of a new individual beginning with the head and proceeding posteriorly under the control and dominance of the head. Newly hatched or newly regenerated normal worms have no posterior zooid. In normal worms the postpharyngeal region does not isolate as a zooid until a definite length is reached; but this happens at a very short length in headless and anophthalmic pieces, and these undergo fission if fed. Large worms consist of several zooids; starvation reduces the zooids and increases head dominance. The zooids disappear when the isolated postpharyngeal region regenerates a new head. Anything which increases the dominance of the head eliminates zooid formation, and vice versa. Decapitation induces fission in small animals.

1910*b*. Analysis of form regulation with the aid of anaesthetics. Biol. Bull., 18:161–73.

1912*c*. Studies on the dynamics of morphogenesis, etc. IV. Certain dynamic factors in the regulatory morphogenesis of *Planaria dorotocephala* in relation to the axial gradient. Jour. Exper. Zoöl., 13:103–52.

1913*a*. Studies on the dynamics of morphogenesis, etc. V. The relation between resistance to depressing agents and rate of metabolism in *Planaria dorotocephala* and its value as a method of investigation. Jour. Exper. Zoöl., 13:103–52.

First full statement (1913*a*) of the technique and results of the direct and indirect susceptibility methods. In the higher concentrations of lethal agents death and disintegration begin at the anterior end and proceed posteriorly. Rate of disintegration is believed to parallel general metabolic rate, and consequently the disintegration gradient is interpreted to demonstrate the existence of a metabolic gradient along the main axis. In dilute concentrations a reverse disintegration gradient is obtained; regions of higher rate acclimate better when adequate time is allowed than regions of lower rate. The retarding effect of anesthetics on regeneration is greater the more posterior the level of the piece; this also indicates the presence of an axial factor, more fully analyzed below.

1913*c*. Studies on the dynamics of morphogenesis, etc. VI. The nature of the axial gradients in *Planaria* and their relation to antero-posterior dominance, polarity, and symmetry. Arch. f. Entw'mech., 37:108–58.

Besides the principal anteroposterior gradient, *Planaria* has also lateromedial and ventrodorsal gradients in susceptibility to lethal solutions. The axiate organism in its simplest terms is a gradient in rate of reaction in a specific colloidal substratum. The region of highest rate dominates or controls other parts and determines the existence and maintenance of the gradient. The axial gradients are the basis of polarity and symmetry. The axial gradient theory alone explains how the organism as a whole can

have a polarity superimposed upon the varied polarities and orientations of its cells and organs. The apical end of the gradient represents, more nearly than any other part, self-differentiation in the specific reaction system of each kind of protoplasm; other parts are the result of correlative differentiation under the influence of the dominant part.

1913*b*. Certain dynamic factors in experimental reproduction and their significance for the problems of reproduction and development. Arch. f. Entw'mech., 35:598–641.

1914*d*. Studies on the dynamics of morphogenesis, etc. VII. The stimulation of pieces by section in *Planaria dorotocephala*. Jour. Exper. Zoöl., 16:413–41.

1914*f*. Studies on the dynamics of morphogenesis, etc. VIII. Dynamic factors in head-determination in *Planaria*. Jour. Exper. Zoöl., 17:61–79.

1916*e*. Studies on the dynamics of morphogenesis, etc. IX. The control of head-form and head frequency in *Planaria* by means of potassium cyanide. Jour. Exper. Zoöl., 21:101–26.

The metabolic rate of pieces of *Planaria* is increased by the mere cutting of them from the body; this increase is greater the shorter the piece and the more posterior its level in the first zooid. This stimulation lasts several hours and then gradually declines. Since this stimulation is greatest in those pieces which show the lowest head frequency in regeneration, there is evidently a relation between head frequency and stimulation of section. By cutting off short anterior pieces from long pieces at various intervals after the latter were cut, it is shown that whether or not a head shall form on a piece is determined within 6–8 hours after section, that is, during the period of greatest stimulation resulting from section. Inhibiting the stimulation of section by putting newly cut pieces into low concentrations of cyanide decreases the head frequency of anterior and increases that of posterior pieces of the first zooid. These facts indicate an antagonism between the activity of the blastema at the anterior cut surface and the old tissue of the piece. If the former (x) dominates, head frequency is high; if the latter (y) dominates, head frequency is low. Head frequency therefore depends on the ratio x/y and can be altered by experimental alteration of either x or y. Cyanide directly inhibits x in anterior pieces and hence decreases head frequency; it prevents the stimulation of y in posterior pieces and hence increases head frequency.

1911*c*. A study of senescence and rejuvenescence based on experiments with *Planaria dorotocephala*. Arch. f. Entw'mech., 31:537–616.

1912*a*. The process of reproduction in organisms. Biol. Bull., 23:1–39.

1913*d*. The asexual cycle of *Planaria velata* in relation to senescence and rejuvenescence. Biol. Bull., 25:181–203.

1914*c*. Starvation, rejuvenescence, and acclimation in *Planaria dorotocephala*. Arch. f. Entw'mech., 38:418–46.

1914*e*. Asexual breeding and prevention of senescence in *Planaria velata*. Biol. Bull., 23:1–39.

1915*c*. Senescence and rejuvenescence. Chicago: University of Chicago Press. Pp. 481.

1916*b*. Age cycles and other periodicities in organisms. Proc. Amer. Philos. Soc., 55:330–39.

1916*d*. Age cycles and other periodicities in organisms. Abstract of 1916*b*. Science, 43:724–25.

1918. Physiological senscence in Hydromedusae. Biol. Bull., 34:49–63.

The theory of, and experiments on, senescence and rejuvenescence are summarized in 1915*c*. The organism is a machine constructed *by* functioning, not *for* function; function and structure are inseparable. Growth and differentiation entail the accumulation of visible structural materials; in lower forms they are reversible processes, and reduction and dedifferentiation are accompanied by removal of structure. Senescence is primarily a decrease in rate of dynamic processes conditioned by the accumulation, differentiation, and other associated changes of the material of the colloid substratum. Rejuvenescence is an increase in rate of dynamic processes conditioned by the changes in the colloid substratum in reduction and dedifferentiation. The direct susceptibility method is presented as a means of measuring roughly the rate of dynamic processes. By this means metabolic rate is found to be higher the younger (or smaller) the animal in various Turbellaria, hydroids, hydromedusae, and ctenophores; higher in the animals resulting from regenerated pieces than in the animals from which the pieces were taken; and higher the more normal the regenerate, i.e., the greater the degree of reconstitution of the piece. The products of fission and budding also resemble young animals metabolically. *Planaria velata* reproduces regularly by fragmenting into small pieces, which encyst and emerge as complete tiny worms. This species can apparently exist indefinitely by this asexual cycle without senescence. The reconstitution involved in regeneration and asexual reproduction are thus rejuvenating. In planarians starvation and accompanying reduction result in increased susceptibility; hence a metabolic condition resembling youth. *P. velata* will exist indefinitely in a youthful state if prevented from growing by properly limited food supply. The axial gradient and the role of physiological isolation from a dominant region in asexual reproduction are discussed. Senescence in plants and lower animals results in decreased dominance of the apical end; hence agamic reproduction with accompanying rejuvenescence. Senescence in man and higher animals appears also to consist of accumulation of structural materials, decline of water content, decrease in growth rate, and decline of metabolic rate; hibernation and starvation probably have some rejuvenating action here. Gamete formation is a process of specialization and differentiation; hence, physiologically gametes are in a state of advanced senescence with accompanying low metabolic rate. Gametes generally form toward the end of the life-cycle, usually after a period of vegetative or asexual reproduction, or of growth. Fertilization and early development are rejuvenating processes. Agamic and gametic reproduction are essentially similar, but the gametes are so far specialized that the peculiarities associated with fertilization are necessary for the initiation in them of rejuvenescence. Senescence is a characteristic and necessary feature of the vegetative life of organisms; rejuvenescence is usually associated with reproduction. In lower forms senescence may be balanced by the rejuvenescence of reproduction, so that there is no progressive senescence or death, and life consists of brief alternating periods of progression and regression; in higher forms senescence of the individual leads to death.

1915*a*. A dynamic conception of the organic individual. Proc. Nat. Acad. Sci., 1:164–72.
1915*d*. Individuality in organisms. Chicago: University of Chicago Press. Pp 213.
1916*a*. The basis of physiological individuality in organisms. Science, N.S., 43:511–23.

Definite form, size, structural pattern, and correlation in activities characterize the organic individual. Particulate and vitalistic theories of individuality or theories based on transport of chemical substances fail to touch the essential problem, the order and unity of the organism. The transmission of dynamic change is presented as the basis of organic individuality. Dynamic change in the last analysis results from the action of external forces on protoplasm; if such action is directed and continued, a more or less permanent gradient of response will be produced in a mass of undifferentiated protoplasm. Such a gradient in the transmission of excitation represents the simplest expression of unity and order in protoplasm and is the simplest axis of polarity and symmetry. Along such a metabolic gradient a relation of dominance and subordination exists, the most active region dominating the others because of its greater speed of reactivity. The evidence for the existence of such metabolic gradients (as tested by susceptibility to lethal agents) in a large number of plants and lower animals and embryos is presented. The facts of regeneration show that the apical region of plants and animals is independent of, and controls, other levels along the major axes to a certain distance. Regeneration is fundamentally the same as embryonic development, consisting in the formation of the apical dominant region first, and then the appearance of other parts in correlation with this. The spatial relations of parts of organisms can be experimentally altered by altering the range of apical dominance, through increasing length of axis, decrease in apical activity, decrease in conductivity, or increase in independence of subordinate parts. Parts thus beyond the range of dominance (physiologically isolated) may produce new gradients and so new individuals. Existing gradients can be abolished; and new gradients, and hence new individuals, established by experimental means. Dominance depends on transmitted change, not on transported substances, and generally increases in range with age, because of the improvement in conducting paths with age.

1914*a*. The axial gradient in ciliate Infusoria. Biol. Bull., 26:36–54.

1914*b*. Susceptibility gradients in animals. Science, N.S., 39:73–76.

1917*b*. The gradient in susceptibility to cyanides in the meridional conducting path of the ctenaphore, *Mnemiopsis*. Amer. Jour. Physiol., 43:87–112.

1919*b*. With L. H. Hyman. The axial gradients in Hydrozoa. I. Hydra. Biol. Bull., 36:183–223.

1919*d*. The axial gradients in Hydrozoa. II. Susceptibility in relation to physiological axes, regions of colony, and stages of development. Biol. Bull., 37:101–25.

Apicobasal disintegration gradients in lethal solutions are reported for a variety of Protozoa, various embryos, hydra and other hydroids, and hydroid colonies; a centroperipheral gradient was found along the nerve of the spider-crab claw; and an aboral-oral gradient along the plate-row of *Mnemiopsis*. Local activities may cause secondary regions of high susceptibility at the stalk of hydra. Lethal agents not only slow down the plates of ctenophores but diminish the transmission, so that the oral parts of plate rows may become physiologically isolated and beat independently.

1915*b*. Axial gradients in the early development of the starfish. Amer. Jour. Physiol., 37:203–19.

1916*c*. Axial susceptibility gradients in the early development of the sea-urchin. Biol. Bull., 30:391–405.

1916*g*. Experimental control and modification of larval development in the sea-urchin in relation to the axial gradients. Jour. Morph., 28:65–133.

1917*c*. Differential susceptibility and differential inhibition in the development of polychaete annelids. Jour. Morph., 30:1–63.

1924*a*. With A. W. Bellamy. Susceptibility in amphibian development. Proc. Roy. Soc. London, B, 96:132–45.

In starfish and sea-urchin eggs, blastulae, and gastrulae and in the early embryos of annelids a gradient is found from animal to vegetative pole; later in annelids and amphibians the posterior growing region becomes prominent as a secondary gradient of activity. In proper concentrations, toxic agents modify development along the lines of either differential inhibition or differential recovery. The former gives microcephalic forms with suppression of the oral lobe in urchins, closer approximation or fusion of the pluteus arms, and progressive obliteration of anteroposterior and mediolateral differentiation; in annelids head and posterior region are inhibited. Differential recovery in sea-urchin larvae results in an increased oral lobe, divergence of the arms, and overdevelopment of anterior and median, as compared to lateral and posterior, parts. These results show that the spatial arrangement and the relative sizes and proportions of parts are controlled by the axial gradients.

1916*f*. Axial ausceptibility gradients in algae. Bot. Gaz., 62:89–114.

1916*h*. Further observations on axial susceptibility gradients in algae. Biol. Bull., 31:419–40.

1917*a*. Susceptibility gradients in the hairs of certain marine algae. Biol. Bull., 32:75–92.

1917*c*. Experimental alteration of the axial gradient in the alga, *Griffithsia bornetiana*. Biol. Bull., 32:213–33.

1919*c*. A study of susceptibility in some Puget Sound algae. Publ. Puget Sound Biol. Sta. 2, No. 48, pp. 249–67.

In a variety of algae of the axiated type, apicobasal disintegration or staining gradients are demonstrated when they are placed in lethal or various colored solutions. A gradient in permeability to the agents is also present. The hairs of various marine algae show an apicobasal gradient when the growth is apical, a basoapical gradient when growth is basal. Obliteration or reversal of the gradient is induced in some cases in lower concentrations of the agents. *Griffithsia*, when confined in a small volume of water, may show a reversal of the normal basipetal gradient and may break up into individual cells. Such cells may grow out new filaments; the apical end of the cell in such cases is whatever end was the most susceptible regardless of the original polarity.

1919*a*. Demonstration of the axial gradients by means of potassium permanganate. Biol. Bull., 36:133–47.

1921*d*. The axial gradients of Hydrozoa. IV. Axial gradients in rate and amount of reduction of potassium permanganate in various hydroids and medusae. Biol. Bull., 41:78–97.

Various organisms placed in weak permanganate solutions show a gradation in color reduction from anterior or oral to posterior or basal end. Medusae give the greatest reduction in marginal and oral regions.

1919*f*. Physiological isolation by low temperature in *Bryophyllum* and other plants. Science, N.S., 50:362–65.

1920*d*. With A. W. Bellamy. Physiological isolation by low temperature in *Bryophyllum*. Bot. Gaz., 70:249–67.

The growing tip of plants dominates lower levels and prevents vegetative growth within a certain distance. A cold block placed below the dominant region does no damage but impedes transmission; it induces the outgrowth of axillary buds in bean seedlings and the formation of plants at the runner tips in *Saxifraga* at much shorter than normal length. A cold block around the petiole of *Bryophyllum* leaves induces the outgrowth of plantlets in the angles of the leaf scallops.

1919*b*. A comparative study of carbon dioxide production during starvation in *Planaria*. Amer. Jour. Physiol., 48:241–57.

1919*c*. The effects of cyanide on carbon dioxide production and on susceptibility to lack of oxygen in *Planaria dorotocephala*. Amer. Jour. Physiol., 48:372–95.

1919*e*. Susceptibility to lack of oxygen during starvation in *Planaria*. Amer. Jour. Physiol., 49:403–19.

1920*a*. With H. L. Robbins. Carbon dioxide production in relation to regeneration in *Planaria dorotocephala*. Biol. Bull., 38:103–22.

Carbon dioxide production, measured by the rate of color change of phenol red, is decreased after exposure to cyanide, decreased in early starvation increased in prolonged starvation, increased by regeneration, and increased temporarily, greatest in the mouth region, by cutting pieces. The death of *Planaria* in cyanide is accelerated by lack of oxygen; an additive effect is indicated. During starvation the susceptibility of the body wall to lack of oxygen increases progressively up to at least 4 months. The reaction to lack of oxygen of such starved animals is the same as that of young fed animals of the same size.

1920*b*. Studies on the dynamics of morphogenesis, etc. X. The head frequency in *Planaria dorotocephala* in relation to age, nutrition, and motor activity. Jour. Exper. Zoöl., 30:403–18.

1921*c*. Studies on the dynamics of morphogenesis, etc. XI. Physiological factors in the development of the planaria head. Jour. Exper. Zoöl., 33:409–33.

Further exposition of the role in head regeneration of the relative rates of activity of the anterior blastema (x) and the old piece (y). Head frequency is lower in young than in old, in starved than in fed, and in pieces kept moving than in quiescent pieces, because the rate of y is relatively higher in the first of each of the paired conditions mentioned. The median region of the head is more susceptible than the lateral regions, and inhibited types of head may show differential recovery after a time with excessive outgrowth of the median region.

1920*c*. Some considerations concerning the nature and origin of physiological gradients. Biol. Bull., 39:147–87.

1921*b*. Certain aspects of the problem of physiological correlation. Amer. Jour. Bot., 8:286–95.

1921*e*. Le Problème de l'intégration physiologique. Scientia, 30:115–26.

1923*c*. Physiological polarity and symmetry in relation to heredity. Genetics, 8:336–54.

1924*b*. The problem of pattern in organisms. I. General survey. Amer. Nat., 58:115–26.

1924*e*. The problem of pattern in organisms. II. The physiological gradients. Amer. Nat., 58:322–36.

1925*d*. The physiological significance of the cephalocaudal differential in vertebrate development. Anat. Rec., 31:369–83.

The chief characteristic of the organism is integration. The problem of organismic integration is one both of pattern and material. The chief pattern of organisms, the axiate pattern, is quantitative and consists in simplest terms of a gradient in rate of the main physiological processes. The various lines of evidence in support of this view are reviewed: evidence from susceptibility differences along the axis of numerous embryos, plants, and animals; of staining and reduction gradients in colored substances; of modifications of development in various agents; differences in electric potential along the axis; axial differences in oxygen consumption and carbon dioxide production. Axiate pattern in the last analysis arises from the differential action of the external world on

protoplasm. The gradients represent physiological conditions under which the hereditary mechanism gives rise to an axiate pattern, and are the physiological basis of polarity and symmetry. They are chiefly significant in initiating orderly series of development events in definite directions. In vertebrate development there is a primary cephalocaudal differential; but later, as in other segmented animals, i.e., annelids, the posterior region shows a caudocephalic gradient; this eventually in vertebrates comes under the control of the head. While the gradients arise in relation to environmental differentials, the specific differentiation and organization resulting from development depend upon the hereditary constitution of the protoplasm concerned.

1921*a*. The origin and development of the nervous system from a physiological viewpoint. Chicago: University of Chicago Press. Pp. xvii+296.

The earlier chapters treat in detail the same topics as above: the problem of pattern, the quantitative gradient as the simplest form of axiate pattern, the evidence for the existence of physiological gradients, dominance and subordination as results of the gradient. The nervous system is the chief morphological and physiological expression of the gradient; the chief aggregations of nervous tissue develop at the high end of the gradient, and further differentiation then proceeds posteriorly along the gradient. Functionally, the simple gradient is an excitation-transmission gradient along which nervous function gradually develops, as seen in the ctenophore plate-row. Other transmissive gradients are exemplified by the heart and digestive tube. Differences in electric potential are characteristic features of the gradients; it is suggested that the outgrowths of neurones and formation of paths in the development of the nervous system are directed by such electric differences. The reflex arc also appears to be related to pattern. Early reflexes are effective mostly in a direction down the gradient. In vertebrate evolution there seems to be an increasing tendency toward upward paths. During vertebrate development the appearance of a secondary caudocephalic gradient in posterior regions may determine short, upward paths.

1923*d*. The axial gradients in Hydrozoa. V. Experimental axial transformations in hydroids. Biol. Bull., 45:181–99.

1923*e*. The general relation between susceptibility and physiological condition. Arch. Int. Med., 32:647–62.

1923*f*. Modification of hydroid development through differential susceptibility. Abstract. Anat. Rec., 26:362–63.

1924*c*. Modification of development in relation to differential susceptibility. Amer. Nat., 58:237–53.

1925*b*. Quantitative factors in the susceptibility of living cells to external agents. Radiology, 4:21–29.

1925*c*. The axial gradients in Hydrozoa. VII. Modification of development through differential susceptibility. Biol. Bull., 48:176–99.

In several general papers the direct and indirect susceptibility methods and their application in experimental work are again reviewed. The basis of the direct susceptibility method is explained thus: "To disturbances producing in any manner alteration beyond the limits of equilibration or tolerance of the system and therefore leading to disruption or irreversible change within a measurable period of time, the susceptibility must vary directly though not necessarily proportionally to the rate of changes characteristic of the system." Thus, a great variety of agents and conditions act in a nonspecific manner and give evidence for the existence of a quantitative gradation in rate of activity along the main axis. Modification of development by differential action of inhibiting agents in relation to the gradient is reviewed and new evidence from hydroids brought forward that the higher levels of the gradient are most inhibited by such agents when acting directly. In hydroids, slightly depressing conditions induce stolons anywhere in the colony and cause hydranth buds to transform into stolons. Planulae are modified from the elongated to a spherical apparently anaxial form; they may recover and then show several polarities, as evidenced by stolon outgrowth. Stolons represent a lower metabolic level than hydranths.

1923*a*. The axial gradients in *Corymorpha palma*. Abstract. Anat. Rec., 24:392.
1925*a*. The axial gradients in Hydrozoa. VI. Embryonic development of hydroids. Biol. Bull., 48:19–36.
1926*a*. With E. Deviney. Contributions to the physiology of *Paramecium caudatum*. Jour. Exper. Zoöl., 43:257–312.
1926*b*. With L. H. Hyman. Studies on the axial gradients of *Corymorpha palma*. I. Respiratory, electric, and reconstitutional gradients. Biol. Generalis, 2:355–74.
1926*d*. Studies on the axial gradients of *Corymorpha palma*. II. Differential susceptibility, penetration, and oxidation-reduction in development and reconstitution. Biol. Generalis, 2:609–30.

Further evidence for axial gradients, using *Paramecium* and hydroid embryos and adults, by means of differential susceptibility, staining and reduction gradients, susceptibility to ultraviolet radiations and ordinary light after sensitization, etc. In *Corymorpha*, axial differences in electric potential, oxygen consumption, carbon dioxide production, and rate and type of reconstitution are reported.

1924*d*. Physiological foundations of behavior. New York: Henry Holt & Co. Pp. xii+330.
1926*c*. Behavior origins from a physiologic point of view. Arch. Neurol. and Psychiat., 15:173–84. (Read before the Fifty-first Annual Meeting of the American Neurological Association, Washington, May, 1925.)
1927*a*. The individual and environment from a physiological viewpoint. Article in The child, the clinic, and the court, pp. 126–55. New York: New Republic, Inc.
1928*c*. Biological foundations of social integration. Publ. Amer. Sociol. Soc., 22:26–42.

A consideration of behavior in the broad sense of reaction to environment. The individual is a behavior pattern in a specific protoplasm. The types of patterns found in organisms—spherical, radial, and bilateral—are reviewed in relation to growth and development in plants and animals. Bilateral symmetry gives the greatest possibilities for differentiation and specialization. The axiate type of pattern (radial, bilateral) originates as an excitation-transmission gradient in protoplasm in response to directed external stimuli. The evidence for the existence of such gradients in relation to the primary axis is reviewed. This evidence comprises: structural axial differences, axial differences in normal development, differential modifications of development by the action of external agents, differential death in various solutions and under various conditions, axial differences in electric potential and respiratory rate, staining and reduction differences. The fact that development can be profoundly modified by the differential action of inhibiting agents with respect to the axial gradient shows the importance of the gradients in normal development. The simple excitation-transmission gradient determines the degree and extent of integration. A relation of dominance and subordination is established—the high, or head, end of the gradient dominates the rest of the axis and acts as pacemaker. Blocking of the transmission or decrease in its effectiveness with distance results in physiological isolation. Twinning and duplication of parts are examples of physiological isolation. The organism evolves as a behavior mechanism, since life is a continuous process of equilibration. Evolution is concerned not with morphological characters but with physiological mechanisms of integration and equilibration. Development is a series of modifications of pattern, and the normal patterns can be readily modified by acting on the gradients. Both fixed and modifiable behavior represent the potentialities of the specific protoplasm. The more fixed behavior is more correlated with the primary physiological gradient than is the more modifiable behavior. In evolution the simple behavior type, dependent on anteroposterior dominance, is replaced by central control; in the higher animals, by the cortex, which is not axiate, receives and correlates impulses from all parts of the body, and is the basis of the great modifiability of behavior seen in higher animals. Human social organization is compared to organismic organization. It consists of a relation of dominance (government) and subordination (the governed). Social integration depends on a dynamic correlation of a transmissive nature among the governed. Leadership or dominance originates directly in environmental conditions and is alterable by environment. Societies and nations are integrated by dominant ideas and are altered by this integration. The lower forms of social integration resemble the lower types of organismic integration in that there is an autocratic relation of dominance and subordination. In higher animals and in higher forms of societies (democracy) there is an escape from this autocracy by means of representative government, corresponding to the development of the cortex in animals.

1926*e*. Experimental control of polarity in *Corymorpha palma*. Proc. Soc. Exper. Biol. and Med., 23:789–90.

1926*g*. Studies on the axial gradients of *Corymorpha palma*. III. Control and modification of polarity and symmetry in reconstitution by differential exposure. Biol. Generalis, 2:771–98.

Regenerating pieces of *Corymorpha* give rise to unipolar, bipolar, and multipolar forms with one, two, or more complete or partial hydranths at one or both ends. The frequency of the various forms varies with level and length of piece. Hydranths arise at points of high metabolic rate; regions in contact tend to develop basal ends. The environmental differential between free surfaces and those in contact thus controls the result and the frequency of the various types of regenerates, and origin of new polarities can be altered by means of this factor.

1926*f*. Physiological control: its protoplasmic basis and its development. Ann. Clin. Med., 4:884–99.

The gradient as the primary expression of polarity and symmetry and the evidence for gradients from differential susceptibility and modification of development are reviewed. The gradient as the basis of physiological control is discussed; pacemakers are examples of control by the high end of the gradient over lower levels. Neoplasms are considered to represent escapes from normal physiological control.

1924*f*. Experimental regression and redevelopment in *Corymorpha palma*. Abstract of 1927*a*. Anat. Rec., 29:98.

1927*a*. Modification of polarity and symmetry in *Corymorpha palma* by means of inhibiting conditions and differential exposure. I. Forms resulting from modification. Jour. Exper. Zoöl., 47:343–83.

1927*b*. Modification of polarity and symmetry, etc. II. Changes in form frequency. Jour. Exper. Zoöl., 48:197–221.

Temporary subjection of reconstituting pieces to inhibiting or depressing agents or conditions more or less obliterates the original polarity; and on return to normal conditions, new polarities arise in such pieces without regard to the original polarity or the cut ends but with reference to the exposed surface. Single or multiple partial or complete hydranths grow out from the exposed surface; single or multiple basal ends, from the surface in contact. The frequency of the various types of regenerates is tabulated. Inhibiting agents decrease the frequency of bipolar forms and increase that of heteropolar forms. Less extreme degrees of inhibition increase unipolar regenerates; more extreme increase multipolar ones.

1923*b*. Experimental eliminations of the larval stage in an ascidian. Abstract, American Association of Anatomists. Anat. Rec., 25:122–23.

1927*c*. Developmental modification and elimination of the larval stage in the ascidian, *Corella willmeriana*. Jour. Morph., 44:467–514.

When the eggs of *Corella*, which normally develop in the atrial chamber of the parent at a pH below that of normal sea water, are removed to normal sea water at early stages, larval development is differentially inhibited, the tail most, the dorsal region less. The regions most inhibited are those showing the highest reducing power in potassium permanganate solutions. The effective natural factor is the CO_2 content of the atrial chamber; but both normal and inhibited development can be produced by other means.

1928*a*. Experimental transformations of bipolar forms in *Corymorpha palma*. Biol. Bull., 54:1–14.

Bipolar forms with a hydranth at each end regress when placed in inhibiting solutions for one or two days, and may then regenerate in normal sea water into a unipolar form. Bipolar forms under depressing conditions may grow out a basal end laterally between the two hydranths, which then face one way, as a double-headed unipolar form.

1928*b*. Physiological polarity and dominance in the holdfast system of *Corymorpha*. Biol. Bull., 54:15–35.

Observations on the formation, structure, and behavior of holdfasts; they show a susceptibility gradient from tip to base and apparently develop under conditions of physiological isolation.

1928*d*. Axial development in aggregations of dissociated cells from *Corymorpha palma*. Physiol. Zoöl., 1:419–61.

Cells and cell groups of the naked stem region, obtained by grinding with sand and then straining through bolting cloth, aggregate into masses which may develop into single or doubled hydranths with stems or imperfect hydranths or irregular tentacles. The polarity is determined by the environmental differential between the exposed and the attached surface of the masses.

1927*d*. Experimental localization of new axes in *Corymorpha palma* without obliteration of the original polarity. Biol. Bull., 53:469–80.

1929*b*. Lateral grafts and incisions as organizers in the hydroid *Corymorpha*. Physiol. Zoöl., 2:342–74.

1932*b*. Determination of new axes by lateral grafts and incisions in *Corymorpha* in relation to the gradient and dominance. Physiol. Zoöl., 5:593–627.

1935*c*. Dominance of hydranths induced by grafts in *Corymorpha*. Jour. Exper. Zoöl., 71:375–87.

A lacerated area in the side of the *Corymorpha* stem may grow out into a hydranth opposite which a basal end may develop, the whole forming an axis at right angles to the original axis. Small grafts implanted into incisions in the stem often grow out into a hydranth composed of both graft and host tissue. The removal of the host hydranth is essential, as its presence inhibits the graft. These grafts are more successful the more distal the region of the stem from which they come and the greater their distance from the host hydranth. The hydranth induced by such grafts dominates a certain length of host stem distal and proximal to it, as indicated by inhibition of regeneration on the host stem, reversal of polarity, or determination of new polarity in the host stem.

1928*e*. The physiological gradients. Sammelreferat. Protoplasma, 5:447–76.

1929*c*. Physiological dominance and physiological isolation in development and reconstitution. Arch. f. Entw'mech., 117:21–66.

General reviews of the gradients, the various methods of demonstrating them, differential modifications of development, dominance and subordination in plants and animals, physiological isolation and its relation to asexual reproduction. The amphibian organizer is probably simply a region of high rate of chemical activity which acts by increasing the metabolic rate of other tissues to the point where they can differentiate into nervous system.

1931*b*. The organism and environment. Peking Nat. Hist. Bull., 5, Part IV: 33–36.

A lecture delivered in China reviewing briefly organismic patterns, their relation to environment, polarity as a quantitative gradient, experimental alterations of the gradient, and determination of polarity and new axes. The essential features of the organism are regarded as a reaction of protoplasm to environment.

1929*a*. Senescence and rejuvenescence from a biological viewpoint. Bull. N.Y. Acad. Med., 5:111–13.

1930*a*. Senescence and rejuvenescence from a biological viewpoint. The Harvey Lectures, Ser. 24, pp. 25–44.

A condensed restatement of the material of 1915*c*.

1930*b*. The susceptibility of *Planaria* to potassium cyanide in relation to hydrion concentration and to certain changes in salt content of the medium. Physiol. Zoöl., 3:90–134.

Differences in the pH, salt content, and osmotic pressure of the medium alter the time of death of *Planaria dorotocephala* and *P. maculata* in cyanide solutions, but in all cases the same susceptibility gradient is observed. The rate of progress of death and presence or absence of disintegration vary greatly with the medium.

1930*c*. With K. M. Beyer. Reconstitution of lateral pieces of *Planaria dorotocephala* and *P. maculata*. Physiol. Zoöl., 3:342–65.

In lateral halves of transverse pieces the original polarity persists in almost all pieces; but in longitudinal strips taken lateral to the ventral nerve cords, one or more heads develop on the median cut surface at right angles to the original polarity in the great majority of cases.

1931*a*. Experimental modification of the scale of organization in the reconstitution of *Tubularia*. Physiol. Zoöl., 4:165–88.

In short pieces, exposure to cyanide solutions decreases the length of the hydranth primordium and so increases the frequency of regenerated hydranths with stems and inhibits the formation of aboral hydranths.

1932*b*. Experimental studies on a Japanese planarian. I. Fission and differential susceptibility. Sci. Repts., Tohoku Univ., Ser. 4, 7:313–45.

In *Planaria gonocephala* rapid growth and decapitation accelerate fission; a third zooid is well developed and the posterior fission product may soon divide again. The anteroposterior susceptibility gradient shows a secondary reversed region in the posterior end. Experiments on the differential effect of cyanide on the two ends of regenerating pieces are presented.

1933*a*. The swimming plate rows of the ctenophore, *Pleurobrachia*, as gradients; with comparative data on other forms. Jour. Comp. Neurol., 57:199–252.

A relation of dominance and subordination exists along each plate-row from aboral to oral end. The aboral end dominates; but any level, if stimulated, can initiate rhythmic impulses and dominate temporarily. Transmission may occur in both directions along a row, and the stronger impulse dominates. The inactivity of a single plate can block the impulse; hence the impulse must be reinitiated or reinforced at each plate. Reversed locomotion is brought about by reversed transmission. Toxic agents show an aboral-oral susceptibility gradient along the plate row; this is interpreted as an excitation-transmission gradient behaving in an all-or-none fashion.

1933*b*. Reconstitution in *Haliclystus auricula* Clark. Sci. Repts., Tohoku Univ., Ser. 4, 8:75–106.

Removal of distal parts is followed by regeneration of tentacles and sense organs and reorganization of the old tissue. Distal pieces bearing sense organs regenerate but little. After oblique cuts, the presence or regeneration of distal sense organs inhibits regeneration at more proximal levels. The distal part of the stalk can reorganize into an umbrella. In toxic solutions the umbrellar margin and manubrial end are the most susceptible and susceptibility decreases proximally along the umbrella.

1933*c*. With Y. WATANABE. The longitudinal gradient in *Stylochus ijimai:* with a critical discussion. Physiol. Zoöl., 6:542–91.

The carbon dioxide production of an axial series of pieces gives a U-shaped gradient, varying somewhat with sexual condition. Carbon dioxide production varies inversely with age. Late embryos have a simple apicobasal susceptibility and reduction gradient, while that of adults is U-shaped, agreeing with the carbon dioxide determinations. Criticisms are answered and discussed.

1934*a*. Differential reduction of methylene blue by living organisms. Proc. Soc. Exper. Biol. and Med., 32:34–36.

1934*b*. The differential reduction of methylene blue by *Paramecium* and other ciliates. Protoplasma, 22:377–94.

1935*b*. With Y. WATANABE. Differential reduction of methylene blue by *Corymorpha palma*. Physiol. Zoöl., 8:395–416.

1936*c*. Differential reduction of vital dyes in the early development of echinoderms. Arch. f. Entw'mech., 135:426–56.

1936*b*. With O. RULON. Differential reduction of vital dyes in certain oligochaetes. Jour. Exper. Zoöl., 74:427–48.

The reduction of methylene blue and janus green in various animals and embryos gives gradients similar to those obtained by other methods. Pieces of *Corymorpha* stems show an apicobasal reduction gradient 5–8 hours after section; irregular results obtain shortly after section. The animal-vegetal reduction gradient of early echinoderm embryos is later disturbed by an increased reducing-power of the primary mesenchyme and the entodermal region before and during gastrulation.

1936*a*. With F. J. Wiercinski. Differential susceptibility of living organisms to supersonic vibrations. Science, 83:604–6.

Supersonic vibrations, a primarily mechanical factor, produce disintegration gradients in *Planaria*, *Tubifex*, and *Hydra*, which are apparently the same as those observed with other agents.

1935*a*. With Y. Watanabe. The head frequency gradient in *Euplanaria dorotocephala*. Physiol. Zoöl., 8:1–40.

At a given level the head frequency decreases with decreasing length of piece, and in short pieces decreases from anterior to posterior end of the first zooid, increasing again in the second zooid. The head frequency rises with delay in cutting short anterior or posterior pieces from the ends of long pieces. The removal of the anterior end of acephalic pieces is followed by regeneration of a normal head, but not if a posterior cut surface is also made. These facts indicate an inhibition of head regeneration emanating from the posterior cut surface, probably from severed nerve paths. Transverse half-cuts with or without removal of tissue near an anterior cut surface retard but do not inhibit head regeneration of that side.

1936*d*. A contribution to the physiology of exogastrulation in echinoderms. Arch. f. Entw'mech., 135:457–93.

The exogastrulae of echinoderms, produced chiefly by LiCl, are discussed from the viewpoint of physiological gradients. Exogastrulation is the result of a high degree of differential inhibition applied at the beginning of development; entoderm and mesenchyme increase at the expense of ectoderm. Applied at late blastula stages, inhibiting agents inhibit the formation of mesenchyme and entoderm, acting directly on the gastric region. In exogastrulae the staining gradients to dyes are inhibited or reversed. Differential recovery expresses itself in thickening and outgrowth of the apical ectoderm and in secondary invaginations.

1937. With O. Rulon. Observations and experiments on developmental pattern in *Pelmatohydra oligactis*. Physiol. Zoöl., 10:1–13.

The pattern of development of tentacles on buds is correlated with that of the parent. Pieces with different anterior levels decrease in regeneration rate and number of regenerated tentacles the more posterior the anterior cut. Buds dominate a certain amount of the adjacent column and inhibit anterior regeneration from cut surfaces near them.

REVIEWS

1906. E. G. Conklin. The organization and cell lineage of the ascidian egg (Jour. Acad. Nat. Sci. Phil., Ser. 2, 13. Part I [1905]: 1–127). Science, N.S., 23:340–44.

1907. T. H. Morgan. Experimental zoölogy. Science, N.S., 26:824–29.

1913. W. Roux, in Verbindung mit Professor C. Cowens, Professor A. Fischel und Professor E. Kuster. Terminologie der Entwicklungsmechanik der Tiere und Pflanzen. Science, N.S., 37:668–69.

1918. J. Loeb. The organism as a whole. Bot. Gaz., 65:274–80.

1925. J. Kemster et F. Prevost. La Matière vivante. Jour. Amer. Med. Assoc., 84:808.

THE FRAGMENTATION OF NAIS PARAGUAYENSIS

(Two figures)

LIBBIE H. HYMAN
Laboratory of Experimental Biology, American Museum of Natural History

THIS report is offered as a contribution to Child's theory of dominance and subordination and physiological isolation in relation to asexual reproduction, among the most fruitful of the conceptions with which he has enriched biology.

INTRODUCTION

1. *The experimental animal.*—The oligochaete which forms the subject of these experiments was accidentally discovered three years ago in aquariums in the Laboratory of Experimental Biology, American Museum of Natural History. My attention was attracted by the fact that fission planes were never seen in the worms although they were obviously multiplying by some asexual method. The worm evidently belonged to the family Naididae, notable among oligochaetes for their method of asexual multiplication by the formation of fission planes partitioning the worm into a chain of two or more zooids, each of which alters into a complete worm with fully developed head and posterior end before breaking loose from the chain. In this particular naid, fission planes were never present; but the finding of many regenerating fragments lead to the suspicion that the animal must reproduce by breaking up into pieces. Accordingly, a worm was isolated into a small dish of water and was found on the next day divided into several regenerating pieces. Other worms were then isolated with the same result. It was thus established that this species of Naididae does not conform to the method of asexual reproduction by fission planes characteristic of the family but instead multiplies by fragmentation. The experimental possibilities of the species were at once perceived, and the animal has been under experimentation continuously since its discovery.

The worm was identified as *Nais paraguayensis* Michaelsen, 1905, known from South America and India. The species is not native to the United States and was probably introduced into the laboratory aquariums with South American plants or fish. The absence of fission planes was noted by the discoverer and other taxonomists on oligochaetes, who suspected reproduction by fragmentation, as mentioned in Stephenson's book (1930, p. 538). The fact of fragmentation was established by Aiyer (1924), who, however, is mistaken in supposing that the worm simply divides in two. Stephenson mentions several other oligochaetes supposed to undergo fragmentation as a regular mode of asexual reproduction, but consultation of the original papers failed to yield any convincing evidence in these cases. Consequently, *N. paraguayensis* constitutes the one well-established case among the oligochaetes of fragmentation as a regular method of asexual reproduction.

It was found that the worm can be cultivated easily and enormous numbers obtained in a very short time. The method of culture is given in detail at the end of the paper.

2. *Normal process of fragmentation.*—When adequately supplied with food, *N. paraguayensis* grows until it reaches a length of 30–40 mm. and then spontaneously frag-

ments into several pieces, usually six to eight, sometimes more. The places at which fragmentation is to occur are not detectable by microscopic examination of the worms. I have not personally witnessed the fragmentation; but a student of Dr. J. W. Buchanan, of Northwestern University, has seen three worms fragment. For some hours preceding the event, the worm coils and uncoils restlessly, but finally twists into a rather tight coil and shortly thereafter constricts and pulls apart. In the observed cases the worms divided into two fragments only. Fragmentation apparently occurs chiefly at night, whether the animals are illuminated or kept in darkness. Further observations on these matters are planned for the future.

The fragments are commonly of approximately equal length and segment number. The head fragment is always longer absolutely and contains more segments than the trunk fragments. It commonly consists of twenty to forty segments in addition to the five specialized head segments. The trunk fragments are of about equal length as a rule, consisting of ten to twenty-five segments. The tail fragment, although commonly but little longer absolutely than the trunk fragments, always contains many more segments than they, because of the gradual decrease in segment size toward the tail. There are usually thirty-five to fifty countable segments in the tail piece, as well as several indefinite segments in process of differentiation. No definite relation between body level and fragment length, beyond those just mentioned, has been noticed; but, in fact, it is rarely possible to know just where in the body axis a given fragment belongs. Figure 1 illustrates the fragments obtained from two worms.

Fragmentation cannot be induced by mechanical means, such as violently squirting the worms in and out of a pipette.

3. *Regeneration.*—Very little study was made of the regeneration process itself. Within a day or two at temperatures of 25°–30° C., each fragment has regenerated a complete new head of five segments and an anal segment. There follows the usual reorganization of the old segments behind the new head into the morphology typical of the postcephalic region (Hyman, 1916). New segments quickly differentiate anterior to the anal segment, and soon the regenerated worm has a long, new tail distinguishable for a few days from the old segments of the original fragment by the light color and smaller size of its segments. Soon the limits of the original fragment are no longer distinguishable; and in 4 or 5 days at room temperature, if food is abundant, the regenerated worms have grown to full length and soon fragment again. The shortest naturally formed fragments seen were seven segments long, but pieces three segments long regenerate perfectly. The head (of five segments) will not regenerate posteriorly unless at least three trunk segments are left attached to it. No cases of atypical or bipolar regenerations have been seen. In a few experiments with pieces from different levels of the anteroposterior axis, no differences in rate or amount of regeneration with respect to level could be detected; but regeneration is so rapid at room temperature that, for a proper study of the role of level in regeneration, regeneration rate would need to be slowed by manipulation of the temperature.

4. *Induction of fragmentation.*—In worms exceeding a length of 20 mm., fragmentation can be induced at will simply by removing the worms from the culture and placing them in dishes of fresh water without food. Since the worms are highly thigmotactic and restless when so isolated, it is desirable to provide them with something into which they can twine. For this purpose a coarse filamentous alga which the worms cannot eat has been kept on hand, and a small washed bit of this has been added to each isolated bunch of

worms. No doubt some thoroughly washed cotton would serve as well. Under such conditions at room temperature (25° C. or higher) most or all of the worms will fragment within 24 hours. Since fragmentation can be induced whenever desired, it becomes practical to experiment with the process of fragmentation itself, and it is to this problem that my attention has been devoted. Of the many possible problems for an attack on which *N. paraguayensis* offers highly suitable material, I have so far confined myself to two questions: What factors control the time of fragmentation; and can the number of fragments into which the worm breaks be experimentally altered?

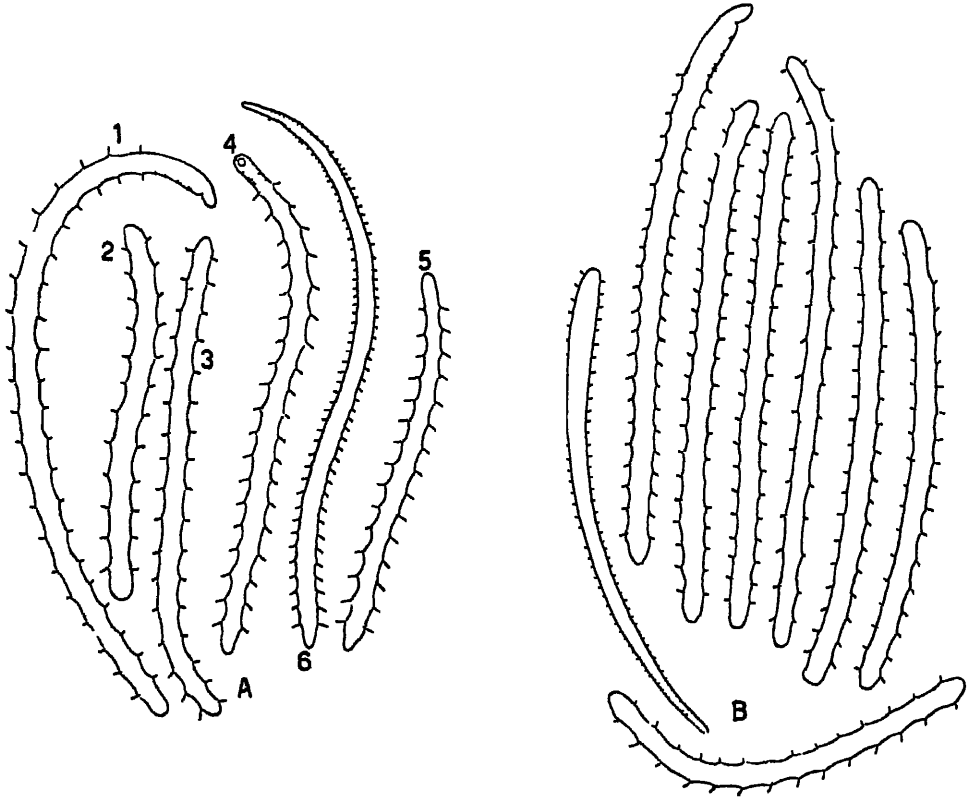


FIG. 1.—Fragments obtained from two worms. Worm *A* fragmented into six pieces, but not simultaneously, so that the levels of the fragments could be ascertained, except fragments 2 and 3, whose relative position is uncertain. The number of segments per fragment is: 1; 21 + 5 head segments; 2, 12; 3, 17; 4, 19; 5, 15; and 6, the tail piece, of over 50 segments. The actual lengths were: 7, 4, 5, 4, 4, and 6.5 mm., respectively. Worm *B* fragmented into eight pieces, the order of which could not be determined; the segment numbers are: 22 + 5, 22, 22, 18, 20, 19, 14, and tail piece of over 40 segments.

THE ROLE OF EXTERNAL AND INTERNAL FACTORS IN THE TIME OF FRAGMENTATION

1. *Theoretical.*—Before proceeding to an account of the experiments, it seems desirable to indicate their theoretical basis. The lines of experiment followed are based upon the well-known conclusions of C. M. Child. In a long series of publications Child has

brought forward experimental evidence for the theory that "the organic individual is fundamentally a dynamic relation of dominance and subordination, associated with and resulting from the establishment of a metabolic gradient or gradients" (1915, p. 88). The region of highest rate of activity of the gradient becomes the apical end of the organism; and this end, by virtue of its high activity, controls and determines the developmental processes at other levels of the major axis. This control, however, has a distance limit, and beyond this limit physiological isolation occurs (Child, 1910, 1911*a*, 1911*b*, 1929), with consequent asexual reproduction in forms capable of regeneration. The range of dominance of the apical end depends on (1) the length of the animal, (2) the metabolic rate of the apical end relative to that of subordinate parts, (3) the efficiency of the conducting paths, and (4) the receptivity of the subordinate parts. Asexual reproduction can be controlled by altering experimentally any of these four factors. For example, incasing the growing-point of a plant or putting a cold block below the growing-point induces the sprouting of latent buds at lower levels of the axis (Child, 1919). According to Child's ideas, any factor which raises the metabolic activity of the head relative to other parts, or lowers the tendency of subordinate parts to become independent, or both, increases the range of dominance of the head and so should inhibit fragmentation in *N. paraguayensis;* and, conversely, any factor which diminishes the activity of the head relative to other parts or stimulates the subordinate parts will induce fragmentation. The following experiments furnish additional evidence for the truth of these generalizations.

2. *Procedure.*—As already indicated, the procedure is simple. Worms of mature length, 30–35 mm., except when the effect of size is under experimentation, are selected from the cultures, rinsed free from culture fluid, and placed in small, covered dishes of clean fresh water with a bit of filamentous alga. At first, Bear Mountain spring water was used for both cultures and experiments, but later the New York City tap water was found perfectly suitable and has since been employed after standing for a few days in a large receptacle. Ten to fifty or sixty worms were placed in each dish, which could then be subjected to any desired condition; or the water in the experimental dishes could be altered in any way wanted. Since fragmentation occurs very rapidly at room temperature, it was necessary in many types of experiments to slow down the process by lowering the temperature. The splendid temperature-regulating equipment of the Laboratory of Experimental Biology permits one to maintain any desired temperature for long periods of time. A temperature of 18° C. has been found most suitable for slowing the fragmentation process.

In counting the number of segments in the fragments, chloretone anesthesia has been employed. A few grains of chloretone are sprinkled on a Stender dish containing the fragments; and in a few minutes the latter become motionless, so that their segment number is easily determined. If the worms are not kept too long under chloretone, they are uninjured by the exposure.

The limitations of space do not permit a complete presentation of the numerous data accumulated. Preliminary experiments, experiments involving fewer than ten worms per dish, and the data on matters on which a negative or uncertain result was obtained have been omitted.

3. *The effect of size on fragmentation.*—It is a common observation that animals do not undergo fission or budding until they reach a certain size. In terms of Child's theories, asexual reproduction occurs when the posterior levels of the organism, because of in-

crease in length, get beyond the range of effective dominance of the head and so become physiologically isolated.

In *N. paraguayensis*, as in other lower invertebrates, the readiness to undergo asexual reproduction is related to size when other factors are constant. Worms of four different sizes were isolated, and the time of fragmentation recorded (Table 1). As readily seen from the table, the longest worms (30–35 mm.) fragment first, the majority within 1 day, the remainder within 2 days, after isolation. The worms of the next size category (20–25 mm.) fragment next; in some cases there is little, if any, difference observable in the time of fragmentation between them and the largest worms at high temperatures, such as 27° C. (Exper. 29, Table 1); but the difference becomes evident when the temperature is lowered (Exper. 53, Table 1). Worms 15–20 mm. long are noticeably slow to fragment, but most accomplish the act if given time enough. The smallest worms, 8–15 mm. long, often fail altogether to fragment; but the larger worms in this category may fragment after several days. Worms 8–10 mm. long were never observed to divide on isolation; such worms are, of course, in process of regulation to normal proportions.

TABLE 1

EFFECT OF SIZE ON THE TIME OF FRAGMENTATION AFTER ISOLATION

Numbers show the number of worms fragmented on day of observation

Exp. No.	Temp. (° C.)	No. of Worms	Days	Length of Worm (Mm.)			
				8–15	15–20	20–25	30–35
27	20	10	5	0	0	9	....
			7	0	7	10	....
53	22	15	1	0	0	2	11
			2	0	3	12	15
			3	0	8	13	....
			6	8	14	15	....
29	27	10	1	0	1	8	7
			2	0	3	10	9
54	27	15	1	0	4	3	10
			2	4	7	12	15
			3	4	12	14	....
			6	10	12	15	....

The relation of size to fragmentation was so evident that relatively few experiments were performed on the matter.

Worms of 15-mm. length fragment into two pieces; those 20 mm. long, into three or four pieces; and the number of fragments increases with further growth in length. The actual length of fragments is, of course, shorter the smaller the worm; but it appears that the number of segments per fragment is not altered by size alone. However, this matter has not been fully investigated as yet. It is thought that a study of the segment number of fragments in relation to size will throw some light on the stage at which the fragmentation levels become fixed and the order of their fixation. Since the worms add on new segments only in front of the anal segment, it is evident that a study of the number of segments in the head fragment, for instance, in worms of different sizes, will reveal whether the fragmentation level for the head fragment is fixed at an early age.

4. *The effect of temperature on the time of fragmentation after isolation.*—The time at which mature worms (30–35 mm.) will fragment after isolation in fresh water depends upon the temperature. At 25–30° C., 75–100 per cent of the worms will undergo fragmentation within 24 hours, and the remainder will fragment by the second day. At 22°–23° C., many—sometimes most—will fragment within 24 hours, the remainder by the second or third day after isolation. At 18°–19° C. some fragmentation occurs within 2 or 3 days, but the majority require 3 or more days. At 16°–17° C. fragmentation is much delayed. Generally no worms fragment before 3 or 4 days, and often only 50 per cent will have fragmented within 5 or 6 days. Frequently some worms remain intact for many days at such temperatures. At 15° C. the fragmentation is very slow, and ordinarily does not begin until 5 or 6 days after isolation. Usually the majority of the worms fail altogether to fragment under such conditions. At 13° C. fragmentation is altogether inhibited, and at temperatures lower than this death generally ensues in a few days. Worms which do not fragment within 5 or 6 days after isolation commonly fail to fragment altogether; a factor of starvation is probably involved here but has not been investigated.

As already mentioned, lowering the temperature constitutes a valuable means of slowing the fragmentation and so magnifying the differences in the action of conditions other than temperature.

5. *The effect of pH on the time of fragmentation.*—The lettuce cultures in which *Nais* is grown have, on reaching equilibrium, a pH of about 7.3. The Bear Mountain spring water, used at first for cultures and experiments, has a pH of 6.9 immediately after the bottles are opened, but rises to 7.8 on standing, presumably through escape of CO^2. The New York City tap water, which was adopted later for cultures and experiments has, on standing, a pH of 7.8. It became necessary to know whether small variations in pH were of any significance in fragmentation experiments and what, in general, is the action of pH on the time of fragmentation. Fully grown worms (30–35 mm.) were isolated into dishes of water having a range of pH from 4 to 9, obtained by adding appropriate amounts of acid or alkali to the water used. The pH was determined by comparison with Lamotte color standards. In each experiment from 17 to 30 worms were put into each pH, but the whole range mentioned was not covered in every experiment. The bunch of worms in each dish was rinsed two or three times with water of the desired pH, and the dish then filled nearly full and covered. To slow down the fragmentation process, all pH experiments were conducted at about 18° C. The dishes were examined after 2 or 3 days, and the number of worms fragmented in each determined by counting the unfragmented individuals. During such an experiment the pH in the experimental dishes alters; the acid water becomes less acid, the alkaline water less alkaline. Possibly a more precise result could be obtained by changing the water frequently during the experiment, but the results secured did not appear to justify more refined experimentation. The data, presented in Table 2, show that extremes of pH, namely, an acidity of 4 or an alkalinity greater than 8, delay the fragmentation, as compared with an intermediate pH. No definite repeatable differences were found in the range from 5 to 8.

In some previous experiments with acidity (Hyman, 1925) I found the carbonate content of the water to play a decisive role. In most of the experiments on pH with *Nais*, spring water was used. This has a much higher carbonate content than the New

York City tap water. However, comparisons of the two kinds of water with respect to the effect of pH on the fragmentation of *Nais* failed to show any difference.

6. *Action of potassium cyanide on the time of fragmentation.*—Potassium cyanide and other cyanide compounds are known to depress the oxygen consumption of the majority of animals. In planarians, for instance, the oxygen consumption can be depressed to 10 per cent of the normal by means of cyanide without damage to the animals (Hyman, 1919; Allen, 1919). The cyanides are, therefore, valuable agents for depressing or inhibiting a variety of phenomena. Since the fragmentation process in *Nais* must involve motor activity at the breaking levels, a depressing agent in proper concentration should retard or inhibit fragmentation, by direct action on the breaking regions. The factor of indirect susceptibility may also be involved. According to Child (1913), regions of high metabolic rate, such as the head, are better able to adjust to the lower concentrations of depressing agents than are regions of lower rate. Nonlethal concentrations of cyanide may, therefore, act not only by directly suppressing the act of fragmentation but by increasing the dominance of the head through the greater ability of the head than the body to acclimate.

TABLE 2

ACTION OF pH ON THE TIME OF FRAGMENTATION AFTER ISOLATION

(Temperature, 18° C.)

Exp. No	No. of Worms	pH 4	5	6	7	8	8.5–9
		No. of Worms Fragmented					
82	20	12	....	19	19	20	....
83	18	....	12	11	15	8	....
84	20	....	15	10	9	3	....
85	20	4	7	6	13	0	0
86	21	....	21	10	8	21	....
87	23	15	23	21	16	22	....
88	27	1	17	2	0	6	0
89	17	....	5	8	9	....	4
90	22	4	16	22	20	20	16
91	28	13	24	25	19	25	10
92	30	11	30	24	28	22	1
93	25	14	21	14	20	22	10
94	25	2	21	24	20	....	....
95	30	1	1	17	22	4	4

In a range of concentrations from 1/10,000 to 1/2,000,000 mol., potassium cyanide was found to retard markedly the fragmentation process of *Nais*, when the process is slowed down by suitable lowering of temperature (Table 3). The result is little correlated with the concentration of cyanide except that high concentrations, such as 1/10,000 cyanide (not presented in the table), altogether inhibit fragmentation within the time limits of the experiment; 1/25,000 mol. is also very inhibiting. The action of cyanide is presumably largely a direct one on the fragmentation process. The worms in cyanide do not differ in appearance or activity from the controls in water.

On theoretical grounds it might be possible to find some concentration of cyanide which would inhibit the head more than the body (without injuring the worms), and hence accelerate fragmentation. In the early experiments with cyanide I thought I had obtained such a result, and so reported in an abstract (Hyman, 1935). Later, however, I was unable, after repeated trials, to verify the early result; and I must conclude that the increased fragmentation seen in several experiments in the cyanided worms, as compared with water controls, was accidental. These early, and more or less preliminary, experiments have been omitted from Table 3, as they could not be verified and as all the later and more extensive experiments gave an inhibiting action of cyanide. The range of concentration which would have an accelerating effect must be very limited; and since, as will be shown below, the physiological state of the worms is subject to much variation, depending on cultural conditions, the effective concentration would probably vary with each lot of worms. These considerations have led me to believe that the experiment in question is impractical.

TABLE 3

ACTION OF POTASSIUM CYANIDE ON THE TIME OF FRAGMENTATION

Temperature 18° C. Concentration of Cyanide in Terms of Molar Strength. Numbers show the number of worms fragmented at the time of observation (2 or 3 days after isolation). Last column gives fragmentation in water control.

Exp. No.	No. of Worms	.00004	.00002	.00001	.000005	.0000025	.000001	.0000005	Water Control
50	40	......	20	23	......	......	......	......	40
61	20	1	8	7	......	......	......	......	11
67	22	4	9	6	......	......	......	......	18
68	23	......	8	8	5	......	......	......	22
69	23	......	......	12	12	16	......	......	21
74	60	3	8	......	......	......	......	......	33
76	42	......	9	13	......	......	......	......	38
77	60	......	......	......	30	......	......	......	53
78	40	......	......	......	......	6	......	......	35
80	40	......	......	......	......	25	19	12	37
81	60	......	......	......	......	9	22	29	59

7. *Effect of decapitation on the time of fragmentation.*—Child has shown (1910, 1911*b*) that decapitation accelerates fission in planarians and induces fission at a smaller size than normally undergoes fission. The induction of fission by decapitation in planarians has been repeatedly verified in class experiments at the University of Chicago and has also been confirmed by Vandel (1922) for European species of planarians. It strongly supports the theory of asexual reproduction as the consequence of escape of posterior levels from the dominance of the head, since decapitation is the simplest method of eliminating the dominance of the head. *Nais paraguayensis* furnishes excellent material for the demonstration of the effect of decapitation on fission. The results are given in Table 4. At the higher temperatures, 25°–27° C., there is little, and sometimes no, difference in the percentage of fragmentation of normal and decapitated worms; but when fragmentation is slowed by lowering the temperature to 15°–22° C., then the accelerating action of decapitation on fragmentation is very evident.

8. *Decapitation in relation to temperature and size.*—Decapitation increases the number of worms which will fragment at lower temperatures, such as 15° C., and is a means of

inducing fragmentation at such temperatures when other temperature problems are being attacked. At 13° C., a temperature at which normal worms will not fragment at all, decapitated worms cast off the tail pieces; and sometimes the trunk may also fragment. The breaking-off of only the tail pieces at such temperatures may indicate that this region is the first to constrict off normally.

Decapitation also accelerates the fragmentation of small worms and may increase the percentage of fragmentation in the smaller sizes. However, decapitation will not induce fragmentation in worms of 10 mm. length.

9. *Behavior of halves and quarters.*—If worms are cut in half, the posterior halves fragment within 1 day at room temperature, while the anterior halves require 2–5 days for fragmentation. If worms are cut into anterior fourth and posterior three-fourths pieces, the latter fragment within a day, while the quarter-pieces fragment very slowly or not at all, beginning with the fifth day after cutting. In the case of anterior three-fourths and posterior quarter-pieces, the former fragment in 2 days, the latter in 1 day or not at all. These findings again show that the presence of the head inhibits fragmentation within a certain distance.

TABLE 4

EFFECT OF DECAPITATION ON THE TIME OF FRAGMENTATION

Last two columns give the number of worms fragmented in normal and decapitated lots.

Exp. No.	Temp. (° C.)	No. of Worms	Days	Normal	Decapitated
16	27	10	1	8	10
29	27	20	1	15	20
51	27	20	1	19	20
56	25	20	1	20	20
57	25	20	1	20	20
57	25	20	1	17	20
51	22	20	1	0	20
51	22	20	1	2	18
53	22	30	1	7	30
16	20	30	1	0	24
51	19	20	1	2	18
51	17	20	1	0	4
56	17	20	1	1	7
			2	8	16
			5	13	20
57	15	40	2	0	4
			4	20	32

EXPERIMENTAL CONTROL OF FRAGMENT LENGTH

The problem here attacked is the question of controlling the number of pieces into which the worm fragments. The matter at issue is: Are the places at which *Nais* constricts into fragments predetermined or can they be altered by environmental means? The problem is evidently a statistical one in which large numbers of worms must be studied. While it would be possible to isolate individual worms and record the number of pieces into which they fragmented, mass experiments have seemed a more practical method of attack. Fully grown worms (30–35 mm.) were allowed to fragment under whatever conditions were desired, and then the length of all the fragments obtained was recorded by counting their segments under chloretone anesthesia. It is true that the length of the segments varies somewhat along the axis, but only in the tail piece is there a marked reduction in segment length. The number of segments in the tail pieces cannot be accurately determined because the last segments are indefinite and not fully differentiated, but an estimate in round numbers of the number of segments in the tail pieces was at first kept. As no evidence was secured that the length of the tail pieces was correlated with any of the procedures tested, the tail pieces were disregarded in later experiments. The head pieces were recorded separately. The data then concern the number of segments in each fragment and are arranged in the tables to give the number of fragments of each segment length obtained in any given experiment. In counting the number of segments in the head pieces, the five head segments were disregarded.

No evidence was obtained of any relation between actual length of the fragments or number of segments per fragment and body level, except that, as already mentioned, the head fragment is always longer and contains more segments than any of the trunk fragments. The trunk fragments, so far as observed, are all of approximately the same length and segment number, without regard to level, and give a normal variation curve with respect to the number of segments. It is these variation curves and the displacements of their modes by experimental means which are the subject of the following experiments.

1. *Fragment length in relation to temperature.*—It seemed probable that fragment length could be altered by the temperature at which fragmentation occurred. Numerous experiments were set up in which lots of worms were placed for fragmentation in different temperatures, usually 25°–27°, 20°–22°, and 15°–16° C. The number of segments was counted in each of the hundreds of fragments obtained, and the results tabulated and studied. The result of all this labor was negative: the length of the fragments cannot be altered by the temperature at which fully grown worms fragment. This finding suggests that the levels at which fragmentation is to occur are already more or less fixed in mature animals.

At low temperatures, such as 15°–16° C., a considerable number of long pieces are obtained, as shown in Table 5. These pieces range from thirty to fifty or more segments in length, while body fragments typically do not exceed twenty-five segments in length. It is evident, however, that these long pieces do not represent a shift in fragment length by low temperature but are simply pieces which failed to carry out the final fragmentation. In short, low temperature partially suppresses the fragmentation process, so that breaking fails to occur at some of the predetermined levels. As already noted, many worms do not fragment at all at 15° C.

2. *Effect on fragment length of culturing worms at different temperatures.*—When placing worms to fragment at different temperatures failed to affect the fragment length, culturing worms at different temperatures seemed the next logical step. According to Child (1910, 1911*b*), culturing at low temperatures increases zooid length in planarians and *Stenostomum;* at high temperatures, decreases zooid length. The explanation given is that the slower growth rate at low temperature permits the dominant region to keep pace with the increase of length, and so extend its range of dominance and retard physiological isolation; while the rapid growth at higher temperature outruns the rate of extension of dominance and so permits earlier isolation of zooids. Van Cleave, how-

TABLE 5

COMPARISON OF FRAGMENT LENGTH AT 25°–27° AND 15°–16° C., SHOWING SUPPRESSION OF FRAGMENTATION AT THE LATTER TEMPERATURE

Numbers show the number of fragments of each length in each lot of worms. Length is expressed as number of segments per fragment.

No. of Segments	Exp. 16		Exp. 18		Exp. 19		Exp. 21		Exp. 24		Exp. 57	
	27°	16°	27°	15°	27°	15°	27°	16°	27°	16°	25°	15°
6	....	....	....	....	....	....	....	2	....	....	....	....
7	2	1	....	....	....	....	....	1	....	....	....	....
8	....	....	....	....	....	....	....	1	....	....	....	....
9	1	....	....	....	....	....	....	....	....	....	....	....
10	....	3	1	....	....	....	....	1	....	....	....	....
11	2	....	2	1	....	....	1	1	....	....	....	....
12	11	2	9	1	....	....	1	6	....	....	....	....
13	9	1	19	2	....	2	13	6	3	....	....	1
14	8	7	30	2	....	1	26	6	6	7	3	....
15	15	3	61	5	3	1	21	13	13	5	4	1
16	4	2	42	5	5	2	17	6	20	6	11	1
17	3	2	24	10	13	3	14	7	16	3	11	2
18	4	4	16	5	18	4	9	8	13	3	13	2
19	4	....	13	3	11	6	1	4	6	7	10	2
20	....	....	9	8	9	4	1	5	2	1	12	1
21	....	2	3	5	8	3	2	1	6	4	9	3
22	....	1	5	2	6	2	....	4	6	....	2	2
23	....	....	1	1	4	2	....	1	3	1	3	2
24	....	1	4	3	....	1	....	....	....	....	....	1
25	....	....	....	1	....	....	....	....	....	....	1	3
26	....	....	....	2	....	....	....	1	....	1	....	1
27	....	....	....	3	....	1	....	....	....	1	....	....
28	....	....	....	2	....	2	....	....	....	1	....	3
29	....	1	....	2	....	3	....	1	....	6	....	....
30	....	1	....	3	....	1	....	1	....	3	....	2
31	....	....	....	....	....	1	....	1	....	1	....	2
32	....	1	....	3	....	1	....	....	....	....	....	2
33	....	....	....	1	....	2	....	1	....	1	....	1
34	....	....	....	....	....	1	....	....	....	1	....	2
35	....	....	....	1	....	2	....	....	....	1	....	4
36	....	....	....	2	....	1	....	....	....	1	....	3
37	....	....	....	1	....	1	....	....	....	1	....	3
38	....	....	....	1	....	2	....	....	....	....	....	2
39	....	....	....	3	....	....	....	....	....	....	....	....
40	....	....	....	1	....	1	....	....	....	....	....	....
41	....	....	....	....	....	....	....	....	....	....	....	2
42	....	....	....	....	....	....	....	....	....	....	....	1
43	....	1	....	1	....	....	....	....	....	....	....	....
44	....	....	....	2	....	....	....	....	....	1	....	....
45	....	....	....	....	....	....	....	....	....	....	....	....
46	....	....	....	1	....	1	....	....	....	1	....	....
47	....	....	....	....	....	1	....	....	....	....	....	....
48	....	....	....	....	....	....	....	....	....	....	....	1
49	....	....	....	1	....	....	....	....	....	....	....	1
50 or more	....	....	....	9	....	2	....	....	....	....	....	3

ever, has recently shown (1937) that in naids the contrary result is obtained with low temperature, namely, a shortening of zooid length. There is also considerable evidence in the literature (reviewed by Van Cleave, where references will be found) that in naids winter temperatures shorten zooid length and summer temperatures increase it. Van Cleave did, however, find that rapid growth at 20°–25° C. in good cultural and nutritional conditions results in short zooids, confirming Child on this point. Poor nutritional and cultural conditions increase zooid length.

The present experiments confirm the results of Van Cleave (although obtained independently) and extend them to another species of naid. Cultures made from new fragments were placed at 27° and 18°–20° C., respectively. Both cultures had ample food supply and were kept in optimal conditions, with frequent changes of water. When the worms had reached full size, lots were isolated from each temperature culture and allowed to fragment at both temperatures. As the worms grew much more rapidly in the higher temperature culture than in the lower, it was not always possible to compare the fragmentation from the two cultures simultaneously. This was done sometimes by letting the warm culture undergo a spontaneous fragmentation. The fragments from this fragmentation grew up to full length about the time the cold culture had first reached fragmentation size.

This experiment has been done in full twice; the main results are given in Table 6. In each experiment, lots of fully grown worms were selected at various times and set to fragment at both 27° and 18°–20° C. Not all of these trials are presented in the table; those omitted gave the same result. It is seen that the fragment length of either the warm or the cold culture remains the same regardless of the temperature in which the fully grown worms are placed for fragmentation, a result which confirms that reached in the preceding section, i.e., that temperature does not affect the fragment length of mature worms. On the other hand, the fragment length is consistently greater in the 27° worms than in the 18°–20° worms. Thus, when *N. paraguayensis* is grown at a relatively low temperature, the fragment length is shortened; and when it is grown at a relatively high temperature, the fragment length is increased, despite the fact that the worms obviously grow much more rapidly at the higher temperature and may pass through two natural fragmentations to one such in the colder culture.

Not only the trunk fragments but also the head fragments are shorter in the cold cultures and longer in the warm cultures. In Experiment 26 the head fragments in the cold culture ranged from eighteen to twenty-seven segments in length, with an average of 24.7, those of the warm culture, from twenty-eight to thirty-six segments in length, average 32.1. In Experiment 40 the head fragments of the cold culture varied from seventeen to thirty segments, average 22.7; of the warm culture, from twenty-four to thirty-eight segments in length, average 28.6.

It should be remarked that it has not been practical to experiment with lower temperatures because of the extremely slow growth at even 15° C.

3. *The effect of aging of the culture on fragment length.*—Throughout the experiments with temperature it was noticed that the average segment number of the fragments, although consistent within any one experiment, varied considerably in experiments carried on at different times, as will be seen in Table 6. At room temperature the average number of segments per fragment might be anything from fifteen to twenty-five. The source of these differences was puzzling for a long time until it was eventually noticed that fragment length bore some relation to the age of the cultures. Experiments were

then set up comparing fragment length in worms from fresh and stale cultures. The data, given in Table 7, show that the fragment length is consistently shorter in worms grown in freshly made cultures than in those grown in old cultures A similar result is obtained by crowding the worms. New fragments were placed in fresh cultures—a small number of fragments in one, a large number in the other. The latter grew noticeably more slowly than the former. The fragment length of fully grown worms from the uncrowded culture is shorter than of similar worms from the crowded culture (Exp. 39, Table 6). The experiment with crowding was performed only once. In all of these experiments, also, the length of the head fragments follows that of the body fragments, being shorter in fresh and longer in aged cultures. These experiments with aging of the culture confirm the findings of Van Cleave (1937), who used another species, *Pristina longiseta.*

TABLE 6

EFFECT OF CULTURING AT DIFFERENT TEMPERATURES ON THE LENGTH OF BODY FRAGMENTS

Numbers show the number of fragments of each length in the different temperatures

No of Segments per Fragment	Exp 26					Exp 40			
	27° at 27°		27° at 20°	20° at 20°	20° at 27°	27° at 27°	27° at 18°	18° at 18°	18° at 27°
7					1				
11				1					
12						1	1		3
13					1	1	1	2	2
14				1	1	3	2	7	7
15				3	2	1	3	6	22
16		1	1	3	6	2	7	15	16
17	1		1	1	9	8	3	9	15
18	1	2		3	12	11	14	1	11
19	1	4			6	3	7	7	1
20	4	3	3	3	6	6	4	1	3
21	8	2	1	1	4	3	4		1
22	5	2	4			2	4		
23	8	2	2		2	3	3		1
24	4		4		1	2	5		
25	1	1	2			5	4		
26	2	2	2	1	1		2		
27	2	1				1	1		
28	1	2				1			
29		1	1						
30									
34				1					
35			1						
36					1				
37							1		
Av	22 2		23 2	19 1	18 4	19 2	19 7	16 2	16 1

The experiments here recorded indicate that in *N. paraguayensis* the levels at which fragmentation will occur are already fixed in fully grown worms but that they can be altered by the conditions under which the fragments grow to maturity. Fresh culture medium and low temperature shorten the fragment length, or, in other words, increase the number of fragments per worm, old culture medium and high temperature lengthen the fragments or decrease the number of fragments per worm. It seems probable that these two factors are really identical, that is, at low temperature the culture ages less rapidly than at high, and the effect is the same as culturing the worms in fresh medium

TABLE 7

THE EFFECT OF AGING OF THE CULTURE AND THE NUMBER OF WORMS PER CULTURE ON FRAGMENT LENGTH

Numbers show the number of fragments of each segment length in samples from various cultures isolated for fragmentation simultaneously at room temperature

No of Segments	Exp 35			Exp 36		Exp 41		Exp 75			Exp 39	
	Old	Old	New	Old	New	Old	New	Old	New	New	Crowded	Uncrowded
9		2			1		1					
10		1		1								
11								1				
12		1	2						1			
13	1	1	4		1		1		2	3		
14			6	2	4		5		9	5		4
15		1	17		5	1	6		11	10		5
16			13	1	8	2	13		11	11		12
17	1		3		13	6	19	1	8	9	4	8
18	6	1	11		17	3	25	4	3	4	1	13
19	3	1	8		9	2	6	2	3	3	10	3
20	18	2	5	3	8	7	8	3	1		12	5
21	9	3	3	1	9	5	7	1			7	2
22	3	2	4	1	5	2	11	4			6	
23	7	4		4	3	3	3	3	1		8	
24	3	8	1	2		7	3	3			3	
25	4	3	1	4		6		7				
26	3	1		3	1	10	1	3			1	
27	2	4		5	1	4		2				
28		3		2		4		2			1	
29		2		2		4		2				
30		1		4		3		1				
31												
32	1	1		2		2		1				
33			1									
34			1			1						
35				1								
36												
37												
38				1								
39				1								
Av	21 4	22 6	17 3	25 5	18 3	23 6	18 2	23 3	15 9	15 9	20 8	17 1

The result is not explicable on the grounds of rate of growth. The worms do certainly grow more rapidly in fresh medium than in old, as can be seen by inspecting the posterior growing region. But the worms also grow much more rapidly at 27° than at 18°. The effect on fragment length of higher temperature is, however, the opposite of that of fresh medium, although both accelerate the growth rate. Similarly, both low temperature and old medium slow down the growth rate but have an opposite effect on fragment length.

The results are not easy to interpret. The available facts seem to indicate that in naids zooid length is controlled chiefly by the concentration of metabolic and putrefactive products in the medium during growth. It seems necessary to postulate a low degree of head dominance and a strong tendency for posterior regions to become physiologically isolated as zooids. When the concentration of metabolic and putrefactive products in the medium is low, the isolation of posterior levels as zooids occurs early and zooids are correspondingly short. A higher concentration of such products retards the tendency of posterior levels to isolate; zooid formation is consequently slower and the zooids longer.

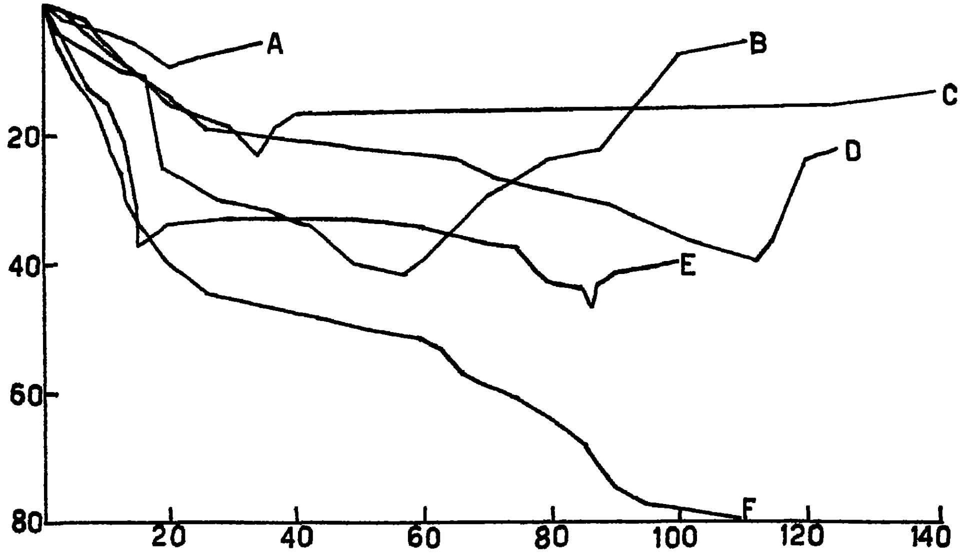


FIG. 2.—Graphs of the disintegration in cyanide of six worms of varying lengths. Segment number on the abscissa; time in minutes on the ordinate.

4. *Disintegration gradient of N. paraguayensis in cyanide.*—In the hope of obtaining some light on physiological conditions in *N. paraguayensis*, the disintegration of a number of individuals in 1/1,000 mol. potassium cyanide was watched and recorded as number of segments disintegrated against time. Some years ago I studied the disintegration gradients of oligochaetes (Hyman, 1916) and found that, in general, they are of the "double" type with disintegration proceeding from both ends toward the middle. The posteroanterior gradient of the posterior part of the body apparently results from the continuous growth of new segments there and seems to have little, if any, integrative value. In *N. paraguayensis* the disintegration gradient shows considerable individual variation, as illustrated in the graphs in Figure 2. Only in the smaller worms is the gradient often of the U-shape found in other oligochaetes (Fig. 2*A*), and some small worms have a continuously descending gradient with no posterior rise. These variations presumably depend upon the rate of growth of the posterior region. In the larger worms, disintegration always begins at the head and progresses posteriorly, giving a rather steep

gradient. This simple descending gradient, however, extends for only twenty to twenty-five segments. Posterior to this level the gradient more or less flattens out, and there is usually a long stretch in which segments disintegrate simultaneously or nearly so (Fig. 2*C*, *D*, *E*). This flattened part of the gradient may comprise the greater part of the worm but may be succeeded by a short posterior region of higher susceptibility (Fig. 2*D*, *E*). Sometimes the larger worms show a typical U-gradient (Fig. 2*B*), and sometimes they give a descending gradient following the flattened region (Fig. 2*F*). Not infrequently the posterior end fails to show any increased susceptibility; this presumably depends upon the rate of growth at the time of testing.

These results indicate that in this worm the head controls only a relatively short length of the axis and that most of the body of the adult animal is physiologically isolated. This isolation apparently begins at a relatively early stage, at which time the fragmentation levels become more or less fixed. No very definite indications of fragmentation levels show up in the disintegration gradients, although probably the sharp dips in the curves in *C*, *D*, and *E* represent such levels. It is also probable that the level where the descending anterior gradient passes into the flattened portion of the gradient is the place where the head fragment separates from the trunk.

SUMMARY

1. *Nais paraguayensis*, a South American fresh-water oligochaete of the family Naididae, reproduces asexually by fragmentation without the formation of typical fission planes. The worms grow to a length of 30–35 mm. and then spontaneously fragment into several pieces, usually six to eight. These pieces regenerate rapidly into complete normal worms.

2. Fragmentation can be induced within 24 hours by isolating fully grown worms into fresh water without food at a temperature of 25°–30° C.

3. The head fragment is typically longer than the body fragments, both absolutely and as to number of segments (typically twenty to thirty, exclusive of the five head segments). The body fragments are approximately equal in length and number of segments, typically ten to twenty. The tail fragment is of about the same length as the body fragments but contains many more segments (mostly thirty to fifty) because of the small size of the most posterior segments.

4. The time of fragmentation after isolation is dependent on size and is more delayed the smaller the worms. Worms 10 mm. or less in length do not fragment.

5. The time of fragmentation after isolation is dependent on temperature and is more delayed the lower the temperature. At 15° C. fragmentation is very slow, and many worms fail altogether to fragment. Fragmentation is usually completely inhibited at 13° C. or lower, and death generally ensues at 10° or lower.

6. Extremes of pH, 4 or below, and above 8 retard the fragmentation. No definite effect was seen in the range from pH 5 to 8.

7. All concentrations of potassium cyanide used (1/10,000 to 1/2,000,000 mol.) retard the fragmentation without affecting activity or appearance of the worms.

8. Decapitation accelerates the fragmentation in all sizes that will fragment and at all temperatures below 25° C.

9. The length of the fragments, that is, the places at which fragmentation occurs, are already fixed in mature worms and could not be altered by any procedures tried.

10. The length of the fragments from mature worms is the same regardless of the temperature at which fragmentation takes place; but at temperatures of 15°–16° C., long pieces may be obtained through suppression of some of the fragmentation levels.

11. The length of the fragments can be altered by the conditions under which fragments grow up to mature worms.

12. Worms cultured throughout their growth at lower temperatures (18°–20° C.) fragment into shorter pieces than those cultured at a higher temperature (25°–27° C.).

13. Worms grown in freshly made cultures fragment into shorter pieces than those grown in old cultures.

14. Worms grown in uncrowded cultures also fragment into shorter pieces than those from crowded cultures.

15. The results obtained under 12, 13, and 14 probably all depend upon one factor, the rate of accumulation of metabolic and putrefactive products in the culture during the growth of the worms. The higher the rate of accumulation of such products, the more slowly are the fragmentation levels established and the greater the distance between them, i.e., the longer the eventual fragments.

16. Disintegration in cyanide gives a simple anteroposterior disintegration gradient only at the anterior end. Over most of the body the gradient is irregular and very shallow, and long stretches of segments disintegrate simultaneously or nearly so. Some indications were seen of a differential susceptibility of fragmentation levels. The posteroanterior gradient typical of the posterior end of aquatic oligochaetes is weakly developed.

17. The results are interpretable on the basis of Child's theory of asexual reproduction as a physiological isolation from a dominant region.

LITERATURE CITED

AIYER, K. S. P. 1924. Reproduction in the aquatic oligochaete, *Nais paraguayensis* Mich. Ann. and Mag. Nat. Hist., Ser. 9, 14:615.

ALLEN, G. D. 1919. Quantitative studies on the rate of respiratory metabolism of *Planaria*. I. The effect of potassium cyanide on the rate of oxygen consumption. Amer. Jour. Physiol., 48:93.

CHILD, C. M. 1910. Physiological isolation of parts and fission in *Planaria*. Arch. f. Entw'mech., 30:159.

———. 1911*a*. Die physiologische Isolation von Teilen des Organismus als Auslosungsfaktor der Bildung neuer Lebewesen und der Restitution. Vorträge und Aufsätze über Entw'mech. (ed. by W. Roux), 11:157.

———. 1911*b*. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. III. The formation of new zooids in *Planaria* and other forms. Jour. Exper. Zoöl., 11:187.

———. 1913. Studies on the dynamics of morphogenesis, etc. V. The relation between resistance to depression agents and rate of metabolism in *Planaria* and its value as a method of investigation. *Ibid.*, 13:103.

———. 1915. Individuality in organisms. Chicago: University of Chicago Press.

———. 1919. Physiological isolation by low temperatures in *Bryophyllum* and other plants. Science, 50:362.

———. 1929. Physiological dominance and physiological isolation in development and reconstitution. Arch. f. Entw'mech., 117:21.

HYMAN, L. H. 1916. An analysis of the process of regeneration in certain microdrilous oligochaetes. Jour. Exper., Zoöl., 20:99.

———. 1919. On the action of certain substances on oxygen consumption. II. Action of potassium cyanide on *Planaria*. Amer. Jour. Physiol., 48:340.

———. 1925. On the action, etc. VI. The action of acids. Biol. Bull., 49:288.

———. 1935. Fragmentation in the naid *Nais paraguayensis* Mich., 1905. Abstract. Anat. Rec., 64, Suppl. 1:79.

MICHAELSEN, W. 1905. Zur Kenntnis der Naididen. Zoologica, 44:350.

STEPHENSON, J. 1930. The Oligochaeta. Oxford: Clarendon Press.

VAN CLEAVE, C. D. 1937. A study of the process of fission in the naid *Pristina longiseta*. Physiol. Zoöl., 10:299.

VANDEL, A. 1922. Recherches expérimentales sur les modes de reproduction des planaires triclades paludicoles. Bull. biol. France et Belgique, 55:344.

APPENDIX

THE CULTURE OF NAIS PARAGUAYENSIS

Shallow vessels are most convenient. Have 1–2 inches of water. Water should be of a type found suitable for other aquatic animals. Put lettuce leaves in water and bring them to a vigorous boil; but do not let boil, as this makes them too mushy. Thin, leafy types of lettuce are preferable, such as romaine and the kind known in New York City as Boston lettuce. Outer leaves discarded by grocery stores are suitable and usually may be had gratis. Place the boiled leaves in the cultures in the proportion of one fair-sized leaf to an 8- or 10-inch dish. Add worms immediately; do not let cultures stand without worms in them. Covering is desirable. Aeration is unnecessary. Temperatures of 25°–30° C. are best for rapid growth; temperatures below 20° should be avoided. As soon as the lettuce leaf is well occupied by worms or evidently going to pieces, add another leaf. Avoid too much lettuce. Disintegrated remains of old leaves should be constantly removed. The worms will commonly abandon old remnants and go on new leaves. Once or twice a week pour off most of the water and replace with fresh water. For best results entirely new cultures should be set up at frequent intervals. Too many worms in a culture inhibit growth; as soon as the worms become abundant, they should be subdivided into fresh cultures. Growth of algae in the culture is undesirable and can be eliminated by putting the cultures in total darkness for a few days. The presence of protozoans, crustaceans, rhabdocoels, etc., in the cultures should be avoided.

Nais paraguayensis is one of the most suitable invertebrates yet discovered for research and class purposes. It can be grown easily and in enormous numbers at negligible expense. It furnishes exceptional material for the study of growth, asexual reproduction, regeneration, action of physical factors and chemical composition of the medium, population biology, conditioning, etc. In addition, it constitutes excellent food for experimental fish and urodele larvae and is easier and more dependable to grow for such purposes than *Daphnia*.

Cultures will gladly be sent to interested zoölogists.

THE EFFECT OF PLANARIAN EXTRACTS AND EXUDATES UPON HEAD REGENERATION IN EUPLANARIA DOROTOCEPHALA

(One figure)

J. WILLIAM BUCHANAN
Department of Zoology, Northwestern University

THE establishment of the principle of organizers, which has been one of the important contributions to knowledge of the physiology of development of the last two decades, has been largely the result of work on vertebrate embryos, principally the amphibian embryo. As is well known, in the amphibian embryo intact living tissues, tissue extracts, tissues killed by heat, and other substances—for example, thyroxin—when applied under suitable conditions elicit responses that in some manner result in differentiations that are beyond the expected developmental fate of surrounding tissues. The literature on regeneration in the invertebrates contains evidence which shows that, with exceptions in the case of regeneration of the planarian head, only intact living tissues are capable of acting as organizers. In *Euplanaria dorotocephala* both living-tissue transplants and external chemical agents induce head formation, the former in the intact animal, the latter in regenerating fragments. This classical experimental animal therefore affords suitable material for further experimental analysis of the nature of inductive influences. The experiments about to be described are continuations and extensions of earlier work (Buchanan, 1922, 1923). They were planned to test the possibility that the anterior region of the planarian, *E. dorotocephala*, from which effective inducing transplants have been made (Santos, 1929) may contain or give off from the living tissue specific agents that induce head regeneration in pieces taken from posterior regions of the animal. The results appear to have an important bearing on the general problem of induction in form regulation and on the specific problem of the nature of the determining conditions in head regeneration in planarian pieces.

The application of the method of mass experiments to regeneration problems by Child (1911) has made it possible to examine with greater accuracy the conditions underlying reconstitution and form regulation. By this method it has been shown that many agents and conditions may cause the development of a head by a piece of a planarian that would otherwise undergo little or no regeneration. This evidence of induction of head formation is found in the greater frequency of occurrence of heads, in various degrees of completeness, among treated lots of pieces, as compared with their frequency in control lots of physiologically similar pieces taken from the same region of the animals and regenerating in water. Among the effective agents are the cyanides, various alcohols, anesthetics, alkaloids, inorganic acids and bases, organic acids, exposure to low temperature, and water conditioned by the animals (Child, 1911, 1912, 1916, 1920; Behre, 1918; Buchanan, 1922, 1923; Hinrichs, 1924; Rustia, 1925; Rulon, 1936, 1937).

Without exception, agents that are effective in increasing head frequency also cause decreases in head frequency when applied in suitable concentrations or intensities. Thus if pieces are treated immediately after section with weak concentrations or intensities, the number of heads or headlike anterior ends formed in a given lot of pieces is significantly greater than among controls. But after treatment with higher concentrations or intensities, the head frequency is significantly less than among controls. The explanation of these remarkable results was first set forth by Child (1911); it is adopted by the writer in two previous papers (1922, 1923) and will be discussed farther on in the present communication.

Induction in planarian regeneration has also been demonstrated by the method of transplantation. If a fragment of living tissue is taken from the head region of a planarian and implanted posterior to the pharynx, the implant exhibits a distinct and positive inducing action, impelling the formation of a head which incorporates both adjacent and transplanted tissue (Santos, 1929). The action is not species specific, for transplants from *E. maculata* induce head formation in *E. dorotocephala*. But homoplastic transplants are effective only if the transplanted tissue is taken from an anterior region and placed in a posterior location.

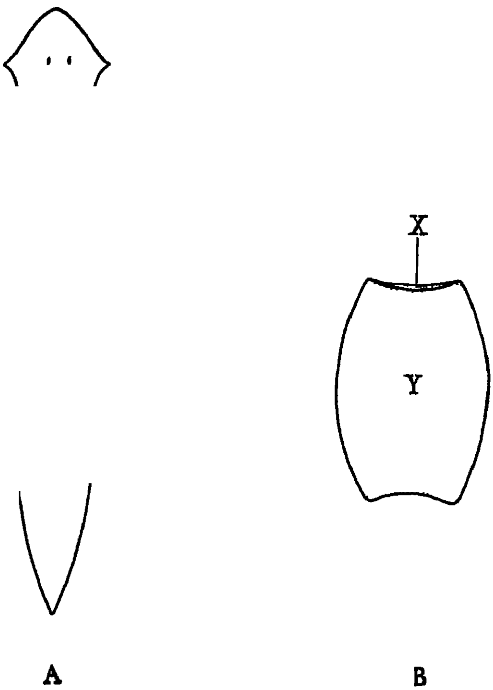


Fig 1.—*A*, diagram of *E dorotocephala* Portion between transverse lines posterior to pharynx employed in experiments *B*, diagram of an isolated piece designating the cells at and near the anterior cut surface as *X*, and the remainder of the piece as *Y*. *Y* also includes the posterior cut surface and adjacent stimulated cells.

MATERIALS AND METHODS

Abundant supplies of *E. dorotocephala* are available in this area. Collections are kept under conditions that are controlled with regard to food supply and temperature, and the selection of animals for experimentation was always made from stocks that had been so controlled for more than a week, usually 3 or 4 weeks after collection. Pieces approximately 1 mm. long were cut from the region just posterior to the pharynx from carefully selected animals of the same length (Fig. 1, *A*). In each experiment a number of such pieces, usually 50 or more, were set aside in water as a control. Similar lots were then cut and treated in various ways, as described farther on. After 10–12 days the number of heads and of other types of regeneration was determined for both control and experimental lots. Differences in the head frequency of the treated lots as compared with the control are attributed to the experimental treatment.

The method is attended by certain difficulties. Two control lots consisting of 50 pieces each, cut consecutively and as nearly as possible under identical conditions, do not always yield the same head frequency; the differences appear to be due to at least two uncontrollable elements: the operator cannot cut with the accuracy necessary to assure that all pieces are taken from exactly the same region of the animals, nor can pieces be cut that do not vary somewhat in length. Moreover, there are undoubtedly distinct individual differences in the regenerative responses of the animals. The writer has repeatedly tested the method by comparing the head frequencies of lots of 50 cut consecutively, and of lots obtained by cutting 100 or more pieces and dividing at random into groups of 50 each. In no case did the range of difference in head frequency between comparable lots exceed 10 per cent. A similar analysis has recently been given by Rulon (1936). Consequently, in the data given below, differences between experimental and control lots exceeding 10 per cent are regarded as significant. Moreover, consistently recurring differences in the same direction of less than 10 per cent may also be regarded as indicative.

A second source of difficulty arises from the fact that whether or not a head will be regenerated by a piece is partially determined within a few hours after the piece is isolated. The cutting of 100 or more small fragments is a tedious and time-consuming task. By the time that the last pieces are cut, the appropriate distribution to flasks completed, and the experimental treatment applied, pieces first cut are several hours older than those last isolated.

In this particular work another difficulty developed because of the great number of animals required for many of the experiments. Although the form is plentiful and easily obtained, large stocks must be kept on hand because selections for experimentation must be made of animals of the same size. Consecutive day-to-day experiments could not be carried out because of lack of numbers of animals of uniform size. Stocks from which selections were made were therefore in the laboratory for different periods. It has been observed that after some weeks in the laboratory the head frequencies of controls increase. Thus repetitions desirable to test the validity of an observation could not always be made upon identical material.

In some American triclads the regulative reaction appears to be of an all-or-none type; either normal heads are regenerated or no regeneration occurs (Buchanan, 1933, 1936). In planarians regenerating fragments form all degrees of anterior regeneration from normal heads to complete absence of anterior tissue. This peculiarity in *E. dorotocephala* permits the detection of differences in the effect of an experimental agent to a finer degree than would otherwise be possible. For such a study it has been found convenient to establish categories of degree of head completeness among regenerating pieces (Child, 1911). Five classes of anterior ends have been distinguished: normal, in which the head is indistinguishable from the heads of collected animals; teratophthalmic, in which the head is normal in shape, or nearly so, but the eyespots are fused to a greater or lesser extent; teratomorphic, in which the head is a rounded protuberance with a single median eye; anophthalmic, in which the head is represented by some anterior outgrowth devoid of eyes; acephalic or headless, in which there is no anterior outgrowth; in this type of regenerate, posterior regeneration is quite complete but the anterior cut surface is simply contracted and filled with scar tissue. Agents which affect head frequency show their effects in several ways: (1) there may be a reduction in the number of regenerated anterior ends of all classes. This occurs when the agent is applied in relatively high concentrations or intensities. (2) There may be an increase in total number of regenerated anterior ends of all classes. Brief exposures usually yield this result. (3) The total number of regenerated anterior ends may not differ from that of the control, but the number in one or another class may be decreased or increased as compared with controls. To evaluate the effects of an agent it is necessary to consider not only the changes in the total number of anterior ends regenerated but also differences between the various classes.

DATA

The most obvious approach to the problem of head induction by specific head-generated substances is to treat isolated pieces with extracts of head tissue. Consequently, in the first series of experiments pieces were taken from the region indicated in Figure 1, *A*, and treated with a simple water extract prepared as follows: heads were cut from 300 animals and placed in a mortar with a few drops of tap water. Grinding was carried on for 5 minutes. Water was then added to make 25 cc., the mass stirred vigorously and then allowed to settle. One hundred animals of approximately the same length were selected and the designated pieces cut. Fifty pieces selected at random for a control were placed in a beaker and covered with 25 cc. of tap water. The head extract was then filtered and poured over the remaining 50 pieces. Both lots were allowed to stand for periods up to 18 hours. The control lot was then removed and placed in 500 cc. of tap water. The experimental lot was washed in several changes of tap water and also placed in 500 cc. of tap water. Water over the pieces was changed every other day. After 10 or more days the regenerates were examined. Three such experiments were carried out, with the results shown in Table 1. An inspection of the data demonstrates that pieces subjected to the head extract regenerated more heads than did the controls.

TABLE 1

HEAD FREQUENCY AMONG REGENERATING PIECES OF *E. dorotocephala* AFTER TREATMENT WITH WATER EXTRACTS OF MASHED PLANARIAN HEADS

(Controls treated with tap water; in per cent)

Lot	No. of Pieces	Duration of Treatment (Hours)	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Headless	Dead
Control.	75	16	17.3	56.0	0.0	9.4	14.6	2.6
Experimental	75	16	26 4	53.3	1.3	5.3	5.3	8.0
Control	50	17	70.0	20.0	0 0	4.0	6.0	0.0
Experimental	50	17	88.0	10.0	0.0	0.0	2.0	0.0
Control	50	14	52.0	24.0	0.0	18.0	6.0	0.0
Experimental	50	14	90 0	6 0	0.0	4.0	0.0	0.0

Attempts were then made to prepare ether and acetone extracts of planarian heads, but the results were not fruitful and the method was abandoned. It was found that when heads were mashed and shaken with ether, no visible deposit was left when the ether evaporated. In fact, when 300 heads were thus treated with 3 cc. of ether, the evaporating ether left no oily stain on white filter paper. Acetone extracts were so minute as to be quite impractical for use.

The data in Table 1 do not answer the question as to whether the increased number of heads regenerated in the experimental lots is due to a specific inductor or to an alteration of the physiological conditions in the isolated pieces, thereby setting up a system conducive to head regeneration. The specific inductive capacity of the head extract was tested as follows: 10 animals, 16 mm. long, were beheaded and individually placed in a Petri dish on ice. After they had become quiet a glass needle, prepared by drawing out a capillary tube, was used to inject a small quantity of head extract into each animal in the region from which the regenerates in Table 1 had been taken. Ten control animals were injured in the same region by the same needle, but without injection. After 11 days each animal was examined under the binocular microscope. In both experimental and control animals several had undergone fission. In the others the injuries had healed without any trace of outgrowths of any sort.

The procedure was varied in another experiment. Ten animals were injured in the region just posterior to the pharynx and then placed in 10 cc. of head extract for 18 hours. Controls similarly injured were placed in the same quantity of water. The results were entirely negative; there was no evidence that the head extract had in any way altered the course of wound-healing or induced any changes whatever in the site of injury.

TABLE 2

HEAD FREQUENCY AMONG REGENERATING PIECES OF *E. dorotocephala* IN RELATION TO VOLUME OF WATER IN CONTAINERS

(In per cent; 50 in each lot)

Lot	Water Volume (Cc.)	Duration of Treatment	Normal	Teratophthalmic	Terato morphic	Anophthalmic	Headless	Dead
Control	500.0	12 days	36	38	0	20	6	0
Experimental	0.2	18 hr.	50	32	2	14	2	0
Control	500.0	14 days	26	50	0	16	8	0
Experimental	0.2	16 hr.	22	40	4	20	10	4
Control	500.0	10 days	18	32	0	36	14	0
Experimental	0.2	17 hr.	18	36	2	30	14	0
Control	500.0	11 days	12	20	2	46	20	0
Experimental	0.2	14 hr.	6	28	2	50	14	0
Control	500.0	12 days	16	32	2	12	34	4
Experimental	1.0	20 hr.	16	46	4	16	18	0

Many years ago Child (1912) showed that when pieces of *E. dorotocephala* were allowed to regenerate in water in which whole animals had been kept, head frequency was appreciably higher compared with controls regenerating in fresh water. This suggests that substances excreted by the intact animal act on head regeneration in the same direction as do extracts of tissues. In the next series of experiments an attempt was made to determine whether or not such exudates are derived solely from the head. Some data were also obtained showing a relation between water volume and head frequency. In Table 2 it appears that keeping the pieces in 0.2 cc. water for periods up to 20 hours after section has no appreciable effect upon head frequency when compared with pieces regenerating for the full period in 10 cc. of water. But in Table 3 it is shown that if heads or posterior ends are added to a 10 cc.-water volume, together with a regenerating piece, a crowding effect occurs. In two cases head frequency is increased and in two others it is decreased by the presence of additional tissue.

Ten experiments on crowding in a small water volume are given in Table 4. Eight show increases in head frequency whether the crowding is due to the addition of heads, tails, or other pieces from the same region of the animal.

From Tables 2, 3, and 4 it appears that (1) short treatment of pieces in exceedingly small volumes of water has no effect upon head regeneration; (2) crowding pieces with either head, tail, or other tissue in small volumes of water for a period up to 24 hours induces increases in head frequency in most cases but may also cause decreases; (3) if crowded in larger volumes of water for the duration of the regenerating period, there is a tendency for head frequency to increase; (4) exudates of heads, tails, or of the pieces themselves induce changes in head frequency.

TABLE 3

HEAD FREQUENCY AMONG REGENERATING PIECES OF *E. dorotocephala* IN RELATION TO WATER VOLUME AND IN THE PRESENCE OF HEADS AND POSTERIOR ENDS

(In per cent)

Lot	No.	Water Volume (Cc.)	Conditions	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Headless	Dead
Control......	50	10	1 piece	44.0	18.0	0	22.0	16.0	0.0
Experimental...	50	10	1 piece 3 heads	54.0	26.0	0	12.0	8.0	0.0
Control.	50	10	1 piece	62.0	22.0	2	4.0	10.0	0.0
Experimental..	50	10	1 piece 3 tails	78.0	10.0	0	2.0	2.0	8.0
Control.......	50	4	1 piece	14.0	34.0	0	18.0	38.0	0.0
Experimental...	50	4	1 piece 1 head	8.0	32.0	4	20.0	32.0	4.0
Control........	80	6	1 piece	3.7	16.2	0	35.0	38.7	0.0
Experimental...	80	6	1 piece 2 heads	0.0	20.0	0	33.7	43.7	2.5

It is then clear that head regeneration may be influenced by the proximity of living planarian tissue if the quantity of water forming the environment is sufficiently small. It is also shown that the controlling influence is derived from living tissue from several regions of the animal. Further evidence of the nature of this influence was then sought. The experiments so far carried out are quite inadequate to determine whether or not planarian tissue may be the source of emanations that influence head frequency; but so far as they go, it is clear that under the conditions of this work the action of living tissue in influencing head regeneration will not operate through glass or quartz ½ mm. in thickness. In each of these experiments 100 pieces were cut as before. As a control, 50 were placed in a pyrex glass beaker with 100 cc. of tap water. The beaker was set in a larger beaker and the space between the beaker walls filled with water. From 150 to 300 freshly cut heads were placed in the water in the outer beaker, resting in the water jacket approximately 5 mm. thick between the two beakers. The remaining 50 pieces were ar-

TABLE 4

HEAD FREQUENCY AMONG REGENERATING PIECES OF *E. dorotocephala* AFTER EXPOSURE TO FRESHLY CUT PLANARIAN HEADS, POSTERIOR ENDS, AND WHEN TWO PIECES FROM THE SAME REGION ARE PRESENT

(Each piece was placed in 0.2 cc. water, and in experimental lots the head or other portion added. After treatment all pieces were removed to five hundred cc. flasks, fifty to each flask. Forty or fifty pieces in each lot.)

Lot	Treatment	Duration (Hr.)	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Headless	Dead
Control	Water	24	40.0	48.5	0	5.7	5.7	0
Experimental	With heads	24	58.8	38.2	0	3.0	0.0	0
Experimental	With tails	24	51.5	42.4	0	6.0	0.0	0
Control	Water	14	6.0	28.0	2	50.0	14.0	0
Experimental	Two pieces	14	8.0	33.0	2	46.0	11.0	0
Control	Water	20	16.0	46.0	4	16.0	18.0	0
Experimental	With heads	20	14.0	34.0	2	18.0	30.0	2
Control	Water	18	32.0	30.0	0	20.0	16.0	2
Experimental	With heads	18	54.0	24.0	2	6.0	10.0	4
Control	Water	24	12.0	48.0	0	20.0	16.0	4
Experimental	With heads	24	28.0	28.0	8	8.0	12.0	16
Control	Water	24	36.0	32.0	0	12.0	16.0	4
Experimental	With heads	24	28.0	56.0	0	4.0	12.0	0
Control	Water	17	18.0	36.0	2	30.0	14.0	0
Experimental	With heads	17	14.0	28.0	2	38.0	16.0	2
Control	Water	17	20.0	36.0	0	26.0	14.0	4
Experimental	With heads	17	32.0	28.0	2	24.0	14.0	0
Control	Water	18	50.0	32.0	2	14.0	2.0	0
Experimental	With heads	18	24.0	40.0	0	20.0	16.0	0

TABLE 5

HEAD FREQUENCY AMONG REGENERATING PIECES OF *E. dorotocephala* WHEN SEPARATED FROM PLANARIAN HEADS BY GLASS AND QUARTZ WALLS ½ MM. THICK

(In per cent)

Lot	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Headless	Dead
Glass (75)	6.6	30.6	5.3	36.6	20	1.3
Quartz (75)	5.2	36.6	2.6	33.3	16	6.6
Glass	14.0	34.0	0.0	20.0	32	0.0
Quartz	14.0	30.0	0.0	18.0	38	0.0

ranged in an identical setup, except that the inner beaker containing the regenerates was made of quartz. The water in all beakers was changed daily.

The experiment was modified as follows: 50 heads were placed in a beaker containing 200 cc. of water and freshly cut heads added. Fifty other pieces were placed in a small quartz beaker containing 100 cc. of water which was then set in a larger beaker containing 100 cc. of water and a like number of heads, 150–300. The results of two of these experiments are given in Table 5. They show no significant differences between the various lots of regenerates. The conclusion is reached that, while head frequency may be increased by adding heads to water containing regenerating pieces, separating the heads and the regenerates by glass or quartz ½ mm. thick prevents the effect of the heads on regeneration.

DISCUSSION

It is shown by the data that tissue extracts, including perhaps products of autolysis, and excretory products of living planarian tissue when applied under suitable conditions cause a given lot of planarian pieces to regenerate a higher percentage of heads or headlike anterior ends than do control lots not so treated. Similar increases in head frequency have been recorded after treatment with cyanides, anesthetics, alkaloids, acids, bases, low oxygen tension, low temperature, conditioned water, and mechanical stimulation (Child, 1911, 1912, 1916, 1920; Buchanan, 1922, 1923; Hinrichs, 1924; Behre, 1918; Rustia, 1925; Rulon, 1936, 1937).

It can hardly be supposed that this extensive group of agents and conditions act upon the pieces in a specific way or confer head-forming properties on cells that otherwise lack such powers. In the first place, the effective agents represent such a wide range of physical and chemical properties that the possibility that all contain a single component which causes heads to develop is precluded. In the second place, it has been shown for most of these agents that they may affect head frequency in either direction, depending on concentration or duration of treatment. One is therefore forced to the conclusion that in inducing head regeneration all these agents act upon some system or condition inherent in the piece, altering such system quantitatively rather than qualitatively.

An interpretation which fits the facts was advanced many years ago. According to Child (1911, and other papers, including Child and Watanabe, 1935), whether or not a new head is regenerated in a piece of *E. dorotocephala* depends upon the relative intensities of the metabolic processes, or certain of them, of two regions of the isolated piece, *X* and *Y* in Figure 1. If the activity of the *X* cells is high in relation to that of the *Y* region, a new head forms. If the activity of the *Y* region is high in relation to that of the head-forming *X* cells, the head-regenerating properties of the *X* cells are more or less inhibited and the new anterior end falls within the categories of more or less complete heads described above and figured in numerous papers by Child, and also by the writer and others.

The importance of the inhibitory action of the *Y* region has recently been emphasized by Child (Child and Watanabe, 1935). It was shown that the sectioning of the nerve cords is an effective factor in determining the inhibitory activity of the *Y* region. But inhibition of head formation by the *X* cells cannot wholly be ascribed to the stimulation of the nerve cords by section, for it has been shown by the writer (Buchanan, 1922, 1923) that those narcotics which act primarily upon nervous tissue are highly effective in increasing head frequency when applied to certain pieces immediately after section, but are quite ineffective when applied 48 hours later. On the other hand, general protoplasmic depressants such as KCN are quite effective in increasing head frequency when applied at either time.

Proofs of the parallelism between physiological activity of the *X* and *Y* regions of the piece and the production of new heads have been established by studies of carbon dioxide output, of oxygen consumption, and of susceptibility to toxic agents (Child, 1914; Robbins and Child, 1920; Hyman, 1919; Buchanan, 1922, 1923). It has been shown that depressants such as the common anesthetics inhibit the metabolic activity of both the *X* and the *Y* regions (Buchanan, 1922). If the *X* cells are more profoundly affected than the *Y* region, then head regeneration is inhibited, as shown by the fact that the head frequency of a given lot of pieces so treated is less than that of a control lot. If the metabolic activity of the *Y* region is more inhibited than that of the *X* cells, a situation is created which makes for increased independence of the *X* cells. Under such conditions in a given lot of pieces the head frequency is greater than in control lots.

The most probable interpretation of the effects on head regeneration of extracts of planarian tissue and of exudates from the intact animal is that such substances are depressants and that their effects on head determination are not different in principle from those of other depressants.

Not much is known concerning the nature of the products of planarian metabolism or of the contents of macerated and autolyzed planarian tissue. Child (1930) showed that *E. dorotocephala* conditions a hypotonic medium, for 10 animals do not disintegrate as rapidly in 125 cc. of distilled water as do 10 like animals in 250 cc. This was confirmed by the writer (Buchanan, 1930), who suggested that the exudates must form osmotically active substances after excretion. But Allee (1933) failed to confirm this suggestion, for he found that water conditioned by *E. dorotocephala* has a slightly lower osmotic pressure than control water taken from the same well. Oesting and Allee (1935) showed that *E. novangliae* excretes minute quantities of calcium into a hypotonic water, and the writer (1933) found that extremely low concentrations of calcium protect the organism against cytolysis by hypotonic agents. But it is extremely doubtful if such minute quantities of calcium induce the states of depression necessary to cause changes in head frequency or that it accounts for the effects shown here. Hyman (1932) showed that the exudates of living planarians do not combine with iodin to any marked extent, but this fact gives little intimation of their physiological effects.

Changes in the water in which *Euplanaria* are living include an increase in carbon dioxide content and a decrease in dissolved oxygen. It was shown by Rulon (1936) that carbon dioxide in suitable concentrations increases the head frequency of regenerating pieces. The depressive effect of insufficient oxygen upon metabolism is too well known to merit comment. Thus either of these two conditions may be called upon to account for the head-frequency increases observed in lots that had been crowded or were in small quantities of water. The fact that plugging the glass capsules caused death after 24 hours is further evidence of the injurious action of interference with gas exchange. But in the case of experiments with filtrates of mashed heads, some other substances must be responsible.

The results reported and reviewed here do not admit of interpretations based upon the specific action of some inductive or organizing substance. This is of considerable importance, for the work of Holtfreter (1933), Waddington, Needham, and Needham (1933), Waddington (1933), Fisher *et al.* (1935), and others on the nature of induction in vertebrate embryos has encouraged the view that organizers exercise control over the development of adjacent tissues by means of specific substances secreted by or diffusing from the controlling elements. According to Holtfreter (1933) the amphibian organizer retains its potency as an inducing agent after a variety of severe treatments, including heating up to 120°. Tissues which show no inducing capacity while living acquire the property when boiled. Fisher and his co-workers (1935) conclude from their work that the effective inducing agent is a nucleo-protein. Apparently the inductive capacity is not species-specific (Holtfreter, 1933; Waddington, 1933; see the discussion by Brensted, 1937).

The diversity of treatment accorded the organizer without destroying its effectiveness, the range of tissues and of tissue extracts that are effective, and numerous other facts call the concept of organizer specificity into question. When the evidence is considered together with the evidence from regeneration and transplantation studies in invertebrates, the facts suggest that the embryo and the regenerate are activable systems which may be influenced quantitatively by a wide variety of agents. At least such conditions appear to be responsible for the experimental alteration of the developmental fate of the X cells in regenerating pieces of *E. dorotocephala*, as shown by the evidence in the present communication and in other papers on experimental alteration of head frequency by Child and his students.

Water extracts of freshly cut planarian heads induce increases in the number of heads regenerated by isolated cross-pieces of *E. dorotocephala* when such extracts are applied for periods less than 24 hours immediately after the sections have been cut.

Head extracts when introduced into the intact animal in a posterior region, or when applied to animals injured in a posterior region, do not induce head formation at the site of injury.

If living heads or posterior ends are included with regenerating pieces in small volumes of water, 0.2 cc., for less than twenty-four hours after cutting, the regenerates show the effects of crowding. In some cases head frequency is increased and in others it is decreased by such treatments.

If the crowding is less intense but continues throughout the regeneration period there is a tendency for head frequency to increase.

Evidence of an effect on head frequency of living planarian heads separated from the regenerates by glass or quartz walls was entirely negative.

These results when considered together with the fact that many other agents and conditions alter head frequency, e.g., low temperature, organic and inorganic acids, bases, anesthetics, and alkaloids, support the conclusion that whether or not a head regenerates in a piece of *E. dorotocephala* depends upon quantitative physiological conditions within the piece; specific action of particular agents cannot be called upon to explain the experimental results.

LITERATURE CITED

ALLEE, W. C. 1933. Studies in animal aggregations. Further analysis of the protective value of biologically conditioned fresh water for the marine turbellarian, *Procerodes*. Physiol. Zoöl., 6:1–21.

BEHRE, E. H. 1918. An experimental study of acclimation to temperature in *Planaria dorotocephala*. Biol. Bull., 35:277–317.

BRENSTED, H. V. 1937. Organisatorstoffe. Protoplasma, 28:123–30.

BUCHANAN, J. W. 1922. The control of head formation in *Planaria* by means of anesthetics. Jour. Exper. Zoöl., 36:1–47.

———. 1923. On the nature of the determining factors in regeneration. *Ibid.*, 37:395–416.

———. 1930. The nature of disintegration gradients. I. The significance of a gradient in susceptibility to distilled water in *Planaria*. *Ibid.*, 57:307–29.

———. 1933. Regeneration in *Phagocata gracilis* (Leidy). Physiol. Zoöl., 6:185–204.

———. 1935. An analysis of physiological states responsible for antero-posterior disintegration in *Planaria dorotocephala*. Protoplasma, 22:497–512.

———. 1936. Notes on an American cave flatworm, *Sphalloplana percaeca* (Packard). Ecology, 17:194–211.

CHILD, C. M. 1911. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. I. The axial gradient in *Planaria dorotocephala* as a limiting factor in regulation. Jour. Exper. Zoöl., 10:265–320.

———. 1912. Studies, etc. IV. Certain dynamic factors in regulatory morphogenesis of *Planaria dorotocephala* in relation to the axial gradient. *Ibid.*, 13:103–52.

———. 1914. Studies, etc. VII. The stimulation of pieces by section in *Planaria dorotocephala*. *Ibid.*, 16:413–41.

———. 1916. Studies, etc. IX. The control of head form and head frequency in *Planaria* by means of potassium cyanide. *Ibid.*, 21:101–25.

———. 1920. Studies, etc. X. Head frequency in *Planaria dorotocephala* in relation to age, nutrition, and motor activity. *Ibid.*, 30:403–18.

———. 1930. The susceptibility of *Planaria* to potassium cyanide in relation to hydrion concentration and to certain changes in salt content of the medium. Physiol. Zoöl., 3:90–134.

CHILD, C. M., and WATANABE, Y. 1935. The head frequency gradient in *Euplanaria dorotocephala*. Physiol. Zoöl., 8:1–40.

FISHER, F. G., WEHMEIER, E., LEHMANN, H., JÜHLING, L., and KULTSCH, K. 1935. Zur Kenntnis der Induktionsmittel in der Embryonal-Entwicklung. Ber. Deutsche Chem. Ges., 68:1196–99.

HINRICHS, M. A. 1924. A study of the physiological effects of caffein upon *Planaria dorotocephala*. Jour. Exper. Zoöl., 40:271–300.

HOLTFRETER, J. 1933. Der Einfluss thermischer, mechanischer, und chemischer Eingriffe auf die Induzierfähigkeit von Triton-Keimteilen. Arch. f. Entw'mech., 132:225–306.

HYMAN, L. H. 1919. Physiological studies on *Planaria*. II. Oxygen consumption in relation to regeneration. Amer. Jour. Physiol., 50:67–81.

———. 1932. The axial respiratory gradient: Experimental and critical. Physiol. Zoöl., 5:566–92.

OESTING, R. B., and ALLEE, W. C. 1935. Further analysis of the protective value of biologically conditioned fresh water for the marine turbellarian *Procerodes wheatlandi*. IV. The effect of calcium. Biol. Bull., 68:314–27.

ROBBINS, H. L., and CHILD, C. M. 1920. Carbon dioxide production in relation to regeneration in *Planaria dorotocephala*. Biol. Bull., 38:103–22.

RULON, O. 1936. The effects of carbon dioxide, the hydrogen ion, calcium, and experimental conditioning on reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl., 9:170–203.

———. 1937. The effects of certain organic acids on reconstitution in *Euplanaria dorotocephala*. *Ibid.*, 10:180–96.

RUSTIA, C. P. 1925. The control of biaxial development in the reconstitution of pieces of *Planaria*. Jour. Exper. Zoöl., 42:111–42.

SANTOS, F. V. 1929. Studies on transplantation in *Planaria*. Biol. Bull., 57:188–97.

WADDINGTON, C. H. 1933. Induction by coagulated organizers in the chick embryo. Nature, 131:275–76.

WADDINGTON, C. H., NEEDHAM, J., and NEEDHAM, D. M. 1933. Beobachtungen über die physikalische-chemische Natur des Organisators. Naturwiss., 21:771–72.

THE MICROSCOPIC ANATOMY OF TWINS AND DOUBLE MONSTERS OF FUNDULUS HETEROCLITUS

MARIE A. HINRICHS
Department of Physiology and Health Education, Southern Illinois Normal University, Carbondale, Illinois

IN A previous series of experimental studies on the modification of development of *Fundulus heteroclitus* by means of ultraviolet radiation, it was possible to produce a considerable number of embryos showing varying degrees of axial duplication (Hinrichs and Genther, 1931).[1]

METHODS

The experimental procedure may be briefly reviewed as follows: fertilized eggs of *F. heteroclitus* were exposed in a shallow layer of sea water for a period of 1 minute, at intervals varying from 5 to 116 minutes after fertilization, to the action of unscreened radiation from a Cooper-Hewitt quartz-mercury-vapor arc, at a distance of 11.5 cm. from the center of the arc. The period most favorable for twin production seemed to be within the 30 minutes just before the appearance of the first cleavage furrow.

For descriptions and sketches of the types found among the 179 experimentally produced twins and double monsters the reader is referred to our earlier report (Hinrichs and Genther, 1931).

It became evident from a study of the gross specimens that it was not always possible to estimate the degree of duplication of organs or parts thereof. Accordingly, the material was sectioned serially for microscopic study.

DATA

Eighty-two sectioned specimens were studied, all of which showed some degree of axial duplication, varying from a complete permanent separation of axes, with subsequent independent development thereof, to somewhat irregular forms with bodies wider than normal, but with little or no obvious external indication of duplication.

Those twins or double monsters which resulted from exposures made as early as from 10 to 15 minutes after fertilization most frequently showed a pronounced effect in the brain region, where there seemed to be a cellular overgrowth with little organization. (Such malformed brains have also been produced in chick embryos [Hinrichs, 1927].) In such embryos the eyes were poorly developed also, sometimes amounting only to a concentration of pigment in the areas where eyes should normally appear. More posteriorly, such a nervous system may become more normal in outline, yet show definite duplication of the neural canal, usually a ventral bifurcation. Others, while obviously greatly irregular in structure—some appearing almost solid with convolutions and masses of rapidly proliferating cells—could still be found to have two distinct divisions each with its own distorted canal—in many instances the mirror image of its mate. Still others appeared wider than normal and were found to have an appended mass of brain tissue more or less irregular in outline and structure, which when followed posteriorly seemed to become part of a partially double neural tube. In such instance, when the duplication appeared ventrally, there also appeared below each half of the cord a complete notochord which was more or less vacuolated and usually wider than a single normal notochord.

---

[1] These studies covered a period of four summers at Woods Hole, Mass., and were completed in 1930. The material thus obtained was prepared for microscopic study by Dr. Ida Genther under a grant from the Radiation Fund of the National Research Council.

Usually the heart and gut and its derivatives were normal in their arrangement, as were other organs. In some instances, however, where the ventral duplication of the neural tube was marked and the notochords parallel, the latter were separated by a mass of muscle tissue which lay just ventral to the neural tube. Such embryos showed a midline depression on the surface.

Embryos of this type have been traced and sketched at intervals throughout development, beginning with the first appearance of any axial delimitation, at which time two parallel equipotential axes could be demonstrated very close to each other.

Another interesting type of twin was formed as a result of the simultaneous development of two equipotential normal antipodean axes. Members of such pairs remained distinct from each other throughout, and on microscopic study showed complete mirror-imaging of organs.

Where the angle between the axes of the developing embryos became less than 180°, the chances for remaining separate entities became less the nearer these axes approached each other on the germ ring, and the chances for subsequent fusion appeared greater. One embryo usually gained advantage over the other, and as development continued the smaller component became differentially more modified. In other words, in this series there was a gradation of inhibitions of the smaller components including embryos differentially inhibited in the midline, so that eyes appeared closer together than normally, or even fused. Sometimes the inhibiting action was great enough to cause the brain to appear irregular and little organized, with an apparent cellular overgrowth, eyes reduced to mere pigment spots, and sooner or later the component fused with the larger more normal component. This is the true "autosite-parasite" type of double monster. In some of these embryos there seemed to be an abnormally large amount of gut tissue.

In some instances the only obvious duplication appearing externally was a large optic cup with double lenses on one side. Microscopically, such embryos showed a malformed brain and posteriorly a duplication of the neural canal, especially in the ventral region. Sometimes such a duplication extended only partway posteriorly, while the rest of the embryo seemed normal, microscopically as well as grossly.

DISCUSSION

From a study of the microscopic examination of serial sections of experimentally produced twins and double monsters, it appears that the means employed in this experiment—namely, ultra-violet radiation—is particularly suited because there is no subsequent dilution factor to be reckoned with as is the case in experiments with chemicals, and the latent effect seems relatively short. Therefore it is possible to interrupt the course of normal development at a given period for only a relatively short interval, and in this way produce a developmental modification of those embryonic parts which at the time are most susceptible to change. Such a period of differential susceptibility has been found immediately to precede the subsequent development of an organ. This suggests an explanation for a condition as described above, in which development is interfered with only in the parts of the embryo developing early, when exposure to radiation is made within the first 15 minutes after fertilization.

The optimum time for interfering with normal axial development seems to come shortly before the two-cell stage in embryonic cell division.

SUMMARY

A microscopic study of serial sections of artificially produced twins and double monsters of *F. heteroclitus* presents new evidence for the following:

1. Completely separated twins and partially fused double monsters which develop equipotentially show a definite mirror image of organs. This seems to be true whether or not the embryos are normal in size and development, and also whether they lie 180° apart on the germ ring or close to each other and parallel, with partial fusion.
2. When one embryo develops more rapidly than another with which it is anatomically joined, it appears to do so at the expense of the smaller embryo. The latter shows a differential inhibition of developing organs. In all such cases there is a definite union, sometimes slight, of the two embryos by means of the nervous system. Also the two axes must be potentially different at the outset.
3. There may be some degree of duplication present—e.g., in the central nervous system—which can be detected only microscopically.
4. Duplication may be present in some parts of the developing embryo and be absent in others, e.g., the heart and gut may be single while parts of the nervous, muscular, and skeletal systems show evidence of duplication.

By this study added evidence is presented:

5. Ultra-violet radiation is an effective means of modifying embryonic development.
6. The effects produced are differential in character, i.e., those parts of the embryo which at the time of exposure are physiologically the most active, are the ones to be modified in development.

LITERATURE CITED

HINRICHS, M. A., 1927. Modification of development on the basis of differential susceptibility to radiation. IV. Chick embryos and ultraviolet radiation. Jour. Exper. Zool., 74:309–42.

HINRICHS, M. A., and GENTHER, I. T., 1931. Ultraviolet radiation and the production of twins and double monsters. Physiol. Zoöl., 4:461–85.

THE INFLUENCE OF THYROXIN ON EYE FORMATION IN PHAGOCATA GRACILIS (HALDEMAN)

(Six figures)

BERNAL R. WEIMER, CHARLES W. PHILLIPS, AND DAGNEY M. ANDERSEN
Bethany College, West Virginia

THE study of the endocrine glands and the physiological effects of their secretions has claimed the attention of investigators for a number of years. The literature in the field of vertebrate endocrinology is voluminous. Moreover, there is gradually accumulating data relating to the effect of vertebrate endocrine secretions on the invertebrates. At present this literature reveals many diverse and apparently contradictory conclusions. Nowikoff (1908) reported an acceleration of the division rate of *Paramecium* when thyroid substance was added to cultures of these animals. A similar effect was reported for *Paramecium* by Budington and Harvey (1915). Shumway (1917) reported that thyroid substance increased the division rate of *Paramecium aurelia* or *P. caudatum* almost to 65 per cent. Abderhalden and Schiffmann (1922) also reported an increase in division rate of *Paramecium* as a result of thyroid extract. Cori (1923) found that thyroxin powder (Parke, Davis & Co.) in alkaline solution accelerated the division rate of *P. putrinum*, but that thyroxin produced no effect. Burge and Estes (1925) found that insulin added to cultures increased sugar metabolism in *Paramecium*, but that thyroxin had no effect. Large amounts of thyroxin apparently were toxic. Woodruff and Swingle (1922), as a result of their studies, concluded that "all evidence from studies on *Paramecium* to the effect that thyroid products accelerate cell anabolism is erroneous." Torrey (1923) and Torrey, Riddle, and Brodie (1924) reported that thyroxin depresses the division rate of *Paramecium*. However, the latter authors report an increase in metabolism as measured by the increase in rate of contraction of the contractile vacuoles.

Some experimental work is reported on the effect of endocrines on arthropods. Hanko (1912) found that *Asellus communis* regenerated missing structures twice as fast in an extract of the hypophysis. Lecithin and glycogen also hasten regeneration in a measure. Kunkel (1918) fed the larvae of *Lucilia* on mammalian thyroid and found a slight retardation of growth of the larvae, and consequently the size of the resulting pupae was smaller. However, animals fed on thyroid tend to pupate more rapidly and have shorter periods of pupation. Romeis and von Dobkiewicz (1921) found that *Calliphora* larvae fed on thyroid develop more slowly than the control animals, and that there was little or no morphological difference between the thyroid-fed animals and the control. Kahn (1921) could find no change in time of metamorphosis of the larvae of *Corethra*, *Ecdyurus*, and *Tenebrio* when fed on desiccated thyroid. Von Herwerden (1923) found a decrease in time of onset of sexual maturity of *Daphnia pulex* when fed on desiccated adrenal cortex. Extract from glands of pregnant animals seemed most effective. Terao and Wakamori (1924) reported that thyroid material increased the number of eggs laid by the second generation of silkworms. Kopéc (1926) found little or no effect induced by the administration of thyroid material to the caterpillar of *Lymantria*, except a diminution in weight.

Ashbel (1935) reported an increase in the respiration of invertebrate tissue when treated with thyroid extract. This increase ranges from 23 per cent in the fresh tissue to an increase of 300 per cent in the desiccated tissue. Synthetic thyroxin, however, had no effect on oxygen consumption. Wulzen (1916) found that planarians fed on pituitary gland showed an increase in fission rate. He also reported that the thyroid has a positive influence on the rate of division and growth of *Planaria maculata*.

There is a wide divergence of opinion among investigators as to the role of metabolic rates in amphibian metamorphosis. There has existed a widespread general assumption that metamorphosis was accompanied by a rise in metabolism (Jahrisch, 1920; Uhlenhuth, 1921; Huxley, 1925; Schulze, 1930, and others). In fact, a rise in metabolism with accompanying metamorphosis has been reported by the investigators mentioned above. However, Etkin (1934), from his experiments and after a critical review of the literature, concludes that "there is no reason at present for ascribing any causative significance in metamorphosis to the supposed metabolism-accelerating influence of the thyroid."

Opinion seems to be divided, however, whether in experimental metamorphosis an agent, such as thyroxin is responsible for an increase in metabolism, or whether metamorphosis itself brings about the increase. Nevertheless, irrespective of the metabolic relations resulting from the treatment of the amphibia with the thyroid extracts or crystalline thyroxin, it has been definitely shown that these substances do hasten amphibian metamorphosis.

This paper presents the results of a series of experiments which show the effect of solutions of thyroxin upon an invertebrate, the common fresh-water flatworm, *Phagocata gracilis* (Haldeman). These experiments are intended to be merely preliminary and to serve as an introduction to other experiments which are now in progress or planned to be carried out in this laboratory in the future.

PROBLEM

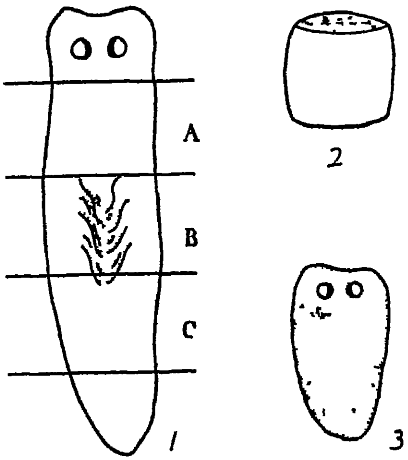
It has long been known by various investigators that the turbellarians have remarkable powers of regeneration. Child and others have compiled a wealth of experimental data with respect to regeneration in *Euplanaria dorotocephala*. The metabolic relations existing in or induced by various agents in cut pieces of *E. dorotocephala* and their effect on regeneration have been further investigated and discussed by Buchanan (1922, 1923). It has been shown that the frequency of head formation in *E. dorotocephala* is dependent upon both the level at which the sections are made and the length of the sections. Further, it has been found that when a section is cut from the animal certain changes in the rate of the metabolic activity of the cut pieces occur. Various reasons are assigned for this increased rate of metabolic activity. It has been shown that cells near the cut surface are stimulated by the act of section. This must be regarded in part as due to wound stimulation. It has been shown also that not only are the cells of the cut surface stimulated by the act of section, but the entire piece is thus affected. This stimulation is usually temporary, subsiding in less than forty-eight hours. It is necessary to emphasize here that not only does wound stimulation affect these cells, but all the cells in the piece are isolated and freed from the dominance of the more anterior regions. Isolation of parts or groups of cells in many organisms brings about rapid differentiation and the initiation of a new organism. It is generally agreed that these processes involve a change in the rate of matabolism.

Experimental evidence has shown that in a piece of an organism undergoing reconstitution there often prevails a high rate of metabolism. Weimer, in unpublished research, has found that reconstitution can be retarded in *Pelmatohydra oligactis* Pallas (Schulze) by lowering the metabolic rate by use of low temperatures.

It was with these considerations in mind that the following preliminary experiments were designed. It was deemed advisable and more accurate to base all observations on the rate of reconstitution of some specific structure rather than on a semigeneralized structure such as the entire anterior end of an animal. For ease and accuracy of observation all data in the following experiments relate to the rate of eye formation.

METHOD

The animals used in these experiments were procured from springs and streams near Bethany. These stock animals were kept in the laboratory in water obtained from the streams in which they were collected. During this period they were not fed. After three days the largest animals of as nearly the same size as possible were selected from this stock. They were placed in culture water in the bottom of inverted finger bowls, and sectioned in the following manner:



FIGS. 1–3.—(1) Sketch of entire animal showing the method of cutting and the designation of levels; (2) appearance of typical cut section; (3) appearance of regenerated piece 10 to 12 days after sectioning.

First, the head region just back of the eyes was cut off and discarded. The main body region of each animal was then sectioned into three equal parts or levels exclusive of the most posterior body region which was discarded. Thus, each piece had two cut surfaces (see Figs. 1–3). The most anterior piece was designated as *A*, the middle piece as *B*, and the posterior piece as *C*.

The *A*, *B*, *C* regions were placed, as soon as cut, in separate finger bowls containing the desired solutions. Usually ten or fifteen animals were used for each series in the experimental solution, and the same number of animals as used in the experimental series was placed in culture water to serve as controls.

The thyroxin solution was made up as follows: 10 mg. of thyroxin (E. R. Squibb & Son) was placed in 1,000 cc. of water taken directly from the water in which the stock animal was living. This solution was shaken and stirred at various intervals. According to Kendall (1929), thyroxin is only slightly soluble in water. At no time have we attempted to analyze the amount of thyroxin present in the solution. After sectioning, the finger bowls containing the control animals and the animals in thyroxin solution were placed on a water table with a temperature of approximately 24° C. The animals were not in direct light.

After a lapse of 10 days, observations were made as to the number of pieces with eyes and the number dead. Observations were made on alternate days until all the animals in the thyroxin solution had developed eyes. In the tabulation of the data it seemed more convenient from the standpoint of comparison to show the results of the observations by percentages rather than by number.

Each time the experiment was repeated, fresh animals, fresh solutions, and the same methods were used. The results of these experiments are summarized in Tables 1, 2, and 3 and in Graphs 1, 2, and 3.

RESULTS

Before discussing the data summarized in the tables, attention is called to the fact that a somewhat similar series of experiments had been made in this laboratory twelve years ago by E. Morton Miller and Irving Tittler. These experiments were of an exploratory nature and were not run in sufficient numbers and with the same uniform technique throughout as that used in the present experiments. However, the results of those experiments agreed essentially with the data presented here.

TABLE 1

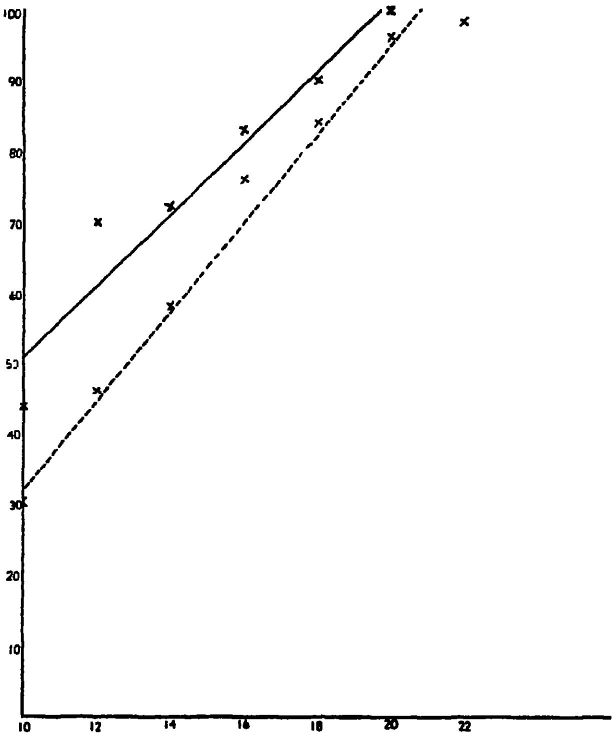
RATE OF EYE FORMATION IN PIECES FROM *A* LEVEL

Observation	No Animals	Thyroxin	Control	Percentage of Increase of Thyroxin over Control
1 (10th day)	50	22—eyes 44 per cent	15—eyes 30 per cent	14
2 (12th day)	50	35—eyes 70 per cent	23—eyes 46 per cent	24
3 (14th day)	50	36—eyes 72 per cent	29—eyes 58 per cent	14
4 (16th day)	50	42—eyes 84 per cent	38—eyes 76 per cent	8
5 (18th day)	50	45—eyes 90 per cent	42—eyes 84 per cent	6
6 (20th day)	50	50—eyes 100 per cent	48—eyes 96 per cent	4
7 (22d day)	50	. . .	49—eyes 98 per cent	2

The study of Table 1 which summarizes the data for the series of experiments on the *A* sections shows that the greatest increase in eye formation of the pieces in thyroxin solution as compared to the controls is found on the tenth, twelfth, and fourteenth day after sectioning. These results are also shown in Graph 1. After that time both the control pieces and the pieces in the thyroxin solution regenerate at about the same rate, although the pieces in thyroxin solution show a differential of 4–8 per cent over the controls.

A study of the data summarized in Table 2 and shown in Graph 2 for the *B* pieces, shows practically the same results as for the *A* pieces except that the pieces in the thyroxin solution show a relative increase in percentage over the control in the later part of the experiment.

The data for *C* level, summarized in Table 3 and depicted in Graph 3, show that there

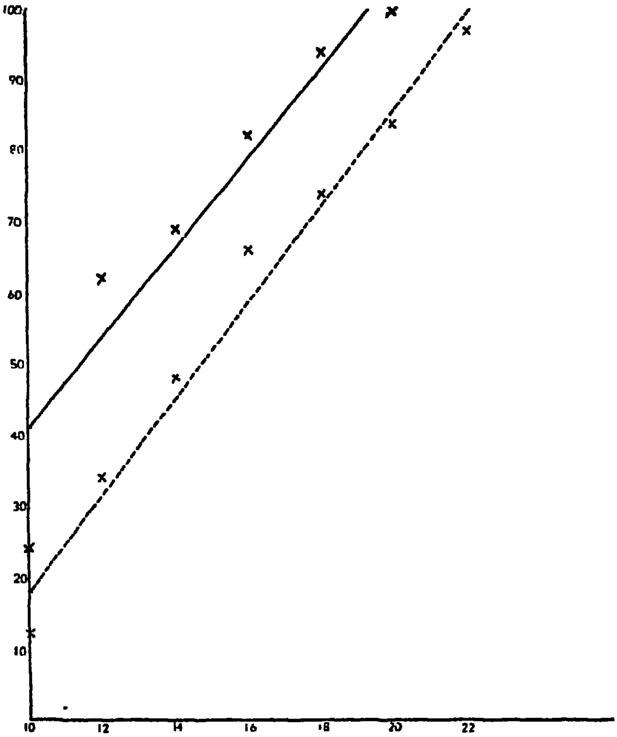


GRAPH 1.—Graph showing the difference in rate of eye formation between pieces from the *A* level in thyroxin solution and those in ordinary water. The ordinates represent the rate of eye formation expressed in percentage and the abscissas represent the time interval in days after sectioning when observations were made. The solid line —— shows the percentage of eye frequency in pieces in thyroxin solution, and the broken line - - - - - - shows the frequency of eye formation in pieces in culture water (control).

TABLE 2

RATE OF EYE FORMATION IN PIECES FROM *B* LEVEL

Observation	No. Animals	Thyroxin	Control	Percentage of Increase of Thyroxin over Control
1 (10th day)	50	12—eyes 24 per cent	6—eyes 12 per cent	12
2 (12th day)	50	31—eyes 62 per cent	17—eyes 34 per cent	28
3 (14th day)	50	37—eyes 74 per cent	24—eyes 48 per cent	26
4 (16th day)	50	41—eyes 82 per cent	33—eyes 66 per cent	16
5 (18th day)	50	47—eyes 94 per cent	37—eyes 74 per cent	20
6 (20th day)	50	50—eyes 100 per cent	42—eyes 84 per cent	16
7 (22d day)	50	....	46—eyes 92 per cent	8



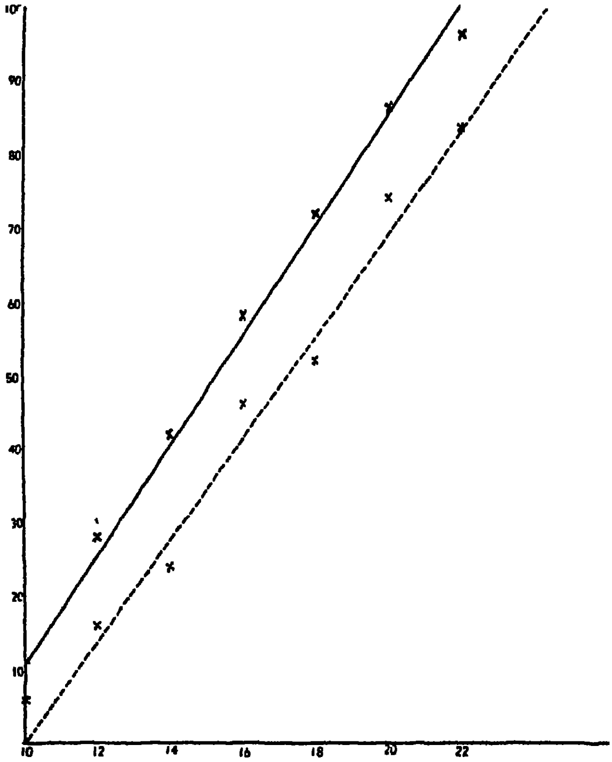
GRAPH 2.—Graph showing the difference in rate of eye formation between pieces from the *B* level in thyroxin solution and those in ordinary water. The ordinates represent the rate of eye formation expressed in percentage and the abscissas represent the time interval in days after sectioning when observations were made. The solid line —— shows the percentage of eye frequency in pieces in thyroxin solution, and the broken line - - - - - - shows the frequency of eye formation in pieces in culture water (control).

TABLE 3

RATE OF EYE FORMATION IN PIECES FROM *C* LEVEL

Observation	No. Animals	Thyroxin	Control	Percentage of Increase of Thyroxin over Control
1 (10th day)	50	3—eyes 6 per cent	....	6
2 (12th day)	50	14—eyes 28 per cent	8—eyes 16 per cent	12
3 (14th day)	50	21—eyes 42 per cent	12—eyes 24 per cent	18
4 (16th day)	50	29—eyes 58 per cent	23—eyes 46 per cent	12
5 (18th day)	50	36—eyes 72 per cent	26—eyes 52 per cent	20
6 (20th day)	50	43—eyes 86 per cent	36—eyes 72 per cent	12
7 (22d day)	50	48—eyes 96 per cent	39—eyes 78 per cent	18

is a percentage increase of the pieces in thyroxin as compared to those of the control. We also find, however, that conditions differ somewhat in this level from the behavior shown for the pieces at the *A* and *B* levels. Thus, the pieces of the *C* level begin to show the greatest percentage differences about the sixteenth day after sectioning. This difference between experimental pieces and controls continues throughout the experiment. The fact that the pieces from the anterior levels regenerate more rapidly than the pieces from the *C* level, in both thyroxin and controls, is in accordance with most observations with respect to regeneration in all *Planaria*, namely, that the rate of regeneration is slower the more posterior the piece. Some exceptions to this rule are noticed in those *Planaria* which have a subordinate zooid present which eventually separates by fission. However, in *P. gracilis* there is no evidence at present to indicate the existence of a second zooid. Further, if a second zooid were present in the *C* level, one would expect an increase in rate of regeneration for these pieces over that shown in *B*.



GRAPH 3.—Graph showing the difference in rate of eye formation between pieces from the *C* level in thyroxin solution and those in ordinary water. The ordinates represent the rate of eye formation expressed in percentage and the abscissas represent the time interval in days after sectioning when observations were made. The solid line —— shows the percentage of eye frequency in pieces in thyroxin solution, and the broken line ------ shows the frequency of eye formation in pieces in culture water (control).

The summary of the total data just presented for all the series is generally true of the various individual experimental series which were run. There were some slight differences between the percentages of the series, but these might be ascribed to such factors as slight differences in the concentration of the thyroxin solutions; fluctuations in temperature; physiological differences of the animals; and other factors. The differences, however, are so slight that the results for the individual series coincide remarkably with those that are summarized in the tables for the total series.

DISCUSSION

The results of the data just presented show quite clearly that pieces of animals placed in a solution of crystalline thyroxin in water will regenerate and reconstitute more rapidly than pieces of animals kept in culture water under the same conditions. A large amount of work done by various investigators indicates quite clearly that thyroxin does increase the rate of metamorphosis in certain vertebrates. Also it has been shown that the rate of metamorphosis in amphibians can be increased by various other compounds, particularly iodin compounds (see summary in Kendall, 1929).

Opinion seems to differ as to the reaction of thyroxin and the iodin compounds as affecting metamorphosis. One view holds that metamorphosis is due to a change in the rate of metabolism of the tissues. Swingle, Helff, and Zwemer (1924) concluded that the increase in metabolism and the hastened onset of amphibian metamorphosis were due to the chemical reactions induced by the thyroxin molecule. Garisch concluded that thyroid brought about its effect through its influence on metabolism. Helff (1926) has found a progressive increase in oxygen consumption for metamorphosing larvae of *R. pipiens*. Kendall (1929), in summarizing a number of other experiments, reports as follows:

> From the results which have been obtained, it cannot be assumed that the metamorphosis is due primarily to a change in the basal metabolism. Neither is it clearly shown that there is an increase in the basal metabolic rate in all forms of amphibia after metamorphosis.

Kendall (1929) further suggests that the influence of thyroxin on the rate of metamorphosis is not due directly to its influence on the rate of energy production.

So far as these experiments are concerned, no attempt has been made to determine whether or not there was an increase in the rate of metabolism. In a letter Hyman suggested that thyroxin here may act as a depressant, which permits the regenerating part to become independent and the regenerating process to proceed more rapidly. The fact that thyroxin was apparently more effective on the *C* pieces would seem to be a confirmatory indication that the rate of regeneration is a result of depression rather than stimulation. With this in mind we have planned further experiments for the very near future. Nor do these experiments exclude the possibility that some slight impurity of the thyroxin may be responsible for the change observed in these experiments. One fact worthy of consideration is that, so far as eye formation is concerned, both the pieces in thyroxin and those in control apparently began this phase of the process of reconstitution after a lapse of approximately the same number of days. However, the rate at which eye formation progresses—once this process has begun—is much higher for those pieces in thyroxin than for those of the controls. This might suggest that, as in amphibian metamorphosis, two processes may be concerned here. Further, the fact must be kept in mind that there may be a wide divergence in the physiological process involved in the metamorphosis of amphibian larvae and the reconstitution of pieces from a flatworm.

SUMMARY

The anterior head region just back of the eyes and the most posterior region of the flatworm *P. gracilis* (Haldeman) were removed, and the remaining body sections were divided in three levels, the most anterior designated as *A*, the middle as *B*, and the posterior as *C*. Some of the pieces, segregated according to levels, were placed in culture water and others in thyroxin solution. Pieces from the *A* level in thyroxin solution showed an increased rate of eye formation in percentage ranging from 2 to 24 per cent (mean value approximately 10 per cent) over those in the control; from the *B* level, an increased rate of 8–28 per cent (mean value approximately 18 per cent) over the control; from the *C* level, an increased rate of 6–20 per cent (mean value approximately 14 per cent) over the control. Thyroxin solution apparently accelerates the rate of eye formation in re-constituting pieces of *P. gracilis*.

LITERATURE CITED

Abderhalden, E., and Schiffmann, O. 1922. Weiter Untersuchung über die von einzelnen Organen hervorgebrachten Substanzen mit spezifischer Wirkung. VII. Chemotaklische Versuche an Paramecien und Untersuchungen über die Geschwindigkeit ihrer Teilung unter den Einfluss von Optonen aus verschiedenen Organen. Arch. ges. Physiol., 94:206.

Ashbel, R., 1935. Action of thyroid extract on the respiration of tissues of invertebrates. Nature, 135:343.

Buchanan, J. W. 1922. The control of head formation in *Planaria* by means of anesthetics. Jour. Exper. Zoöl., 36:1.

———. 1923. On the nature of the determining factors in regeneration. *Ibid.*, 37:395.

Budington, R. H., and Harvey, H. F. 1915. Division rate in ciliate Protozoa as influenced by thyroid constituents. Biol. Bull., 28:304.

Burge, W. E., and Estes, A. M. 1925. The effect of insulin, thyroxin and temperature on the sugar metabolism of *Paramecium*. Jour. Metabol. Res., 7–8:183.

Child, C. M. 1924. Physiological foundations of behavior. Chicago.

Cori, G. T. 1923. The influence of thyroid extracts and thyroxin on the rate of multiplication of *Paramecia*. Amer. Jour. Physiol., 65:295.

Etkin, W. 1934. The phenomena of anuran metamorphosis. II. E. Oxygen consumption during normal metamorphosis. Jour. Physiol. Zoöl., 7.

Hanko, B. 1912. Über den Einfluss einiger Lösungen auf die Hautung, Regeneration und das Wachstum von *Asellus communis*. Arch. f. Entw'mech., 34:477.

Helff, O. M. 1926. Studies on amphibian metamorphosis. II. The oxygen consumption of tadpoles undergoing precocious metamorphosis following treatment with thyroid and di-iodotyrosine. Jour. Exper. Zoöl., 65:69.

Herwerden, M. A. von. 1923. Der Einfluss von kleinen Quantitaten Nebennierenrinde des Rindes auf das Wachstum der Süsswasserschnecke Limaea. Arch. f. mikr. Anat. u. Entw'mech., 98:505.

———. 1923. Der Einfluss der Nebennierenrinde auf das Wachstum und die Fruchtbarkeit von *Daphnia pulex*. *Ibid.*, p. 220.

Huxley, J. 1925. Studies on amphibian metamorphosis II. Proc. Roy. Soc., B, 98:113.

Järisch, A. 1920. Über die Wirkung der Schilddruse auf Kaulquappen. Pflügers Arch., 179:159.

Kahn, R. H. 1921. Über Schilddrüsenfütterung an Wirbellosen. Arch. ges. Physiol., 192:86

Kendall, E. C. 1929. Thyroxine. New York.

Kopćc, S. 1926. Is the insect metamorphosis influenced by thyroid feeding? Biol. Bull., 50:339.

Kunkel, B. W. 1918. The effects of the ductless glands on the development of the flesh flies. Jour. Exper. Zoöl., 26:255.

Nowikoff, M. 1908. Über die Wirkung des Schilddrüsenextraktes und einiger anderer Organstoffe auf Ciliaten. Arch. f. Protistenk., 11.

Oliver, G. O., and Scheaffer, E. A. 1895. On the physiological action of extract of pituitary body and certain other glandular organs. Jour. Physiol., 18.

Romeis, B., and Dobkiewicz, L. von. 1921. Experimentelle Untersuchungen über die Wirkung von

Wirbeltierhormonen auf Wirbellose. I. Der Einfluss von Schilddrüsenfütterung auf Entwicklung und Wachstum der Schmeissfliege (*Calliphora vomitoria*). Arch. Entw'mech., **47**:119.

SCHULZE, W 1930. Der Einfluss der inkretorischen Drusen und des Nervensystems auf Wachstum und Differenzierung. In Bethe *et al.* Handb d. Norm. und Path. Physiol., **16**:II/I:699.

SHUMWAY, W. 1917. The effects of a thyroid diet upon *Paramecium*. Jour. Exper. Zoöl., **22**:529.

SWINGLE, W. W.; HELFF, O. M.; and ZWEMER, R. L. 1924. The effect of thyroxin and its acetyl derivative on amphibians and mammals. Amer. Jour. Physiol., **70**:208.

TERAO, A., and WAKAMORI, N. 1924. Preliminary note on the effects of thyroid feeding on the silkworm. Japan. Med. World, **68**.

TORREY, H. B. 1923. The effect of dissolved thyroxin on the division rate of *Paramecium*. Anat. Rec., **26**:367.

TORREY, H. B., RIDDLE, M. C., and BRODIE, J. L. 1924. Thyroxin as a depressant of the division rate of *Paramecium*. Jour. Gen. Physiol., **7**:449.

UHLENHUTH, E. 1921. The internal secretions in growth and development of amphibians. Amer. Naturalist, **55**:193.

WOODRUFF, L. L., and SWINGLE, W. W. 1922–23. The effects of thyroid products on *Paramecium*. Proc. Soc. Biol. and Med., **20**:386.

WULZEN, R. 1916. Pituitary gland: its effect on growth and fission of planarian worms Jour. Biol. Chem **25**:625.

THE EFFECT OF DEAD OPTIC VESICLE UPON EXPLANTS OF PROSPECTIVE ECTODERM

(Five figures)

C. D. VAN CLEAVE
Department of Anatomy, University of
Pennsylvania, Philadelphia
Pennsylvania

THE circumstances surrounding the formation of the lens vesicle of the eye from the ectoderm under the influence of the underlying optic vesicle suggest that a chemical factor is concerned in this classical case of dependent development. Lewis (1904) discussed the possible chemical nature of the stimulus arising in the optic vesicle and suggested a pertinent approach to the problem: "In the example of the lens reaction it seems evident that the living ectodermal cells must be acted upon, but it is not so evident that the living optic vesicle cells are necessary, as it may be possible to extract from them substances which will give the lens reaction." All investigation of the properties of the dead optic vesicle, however, was destined to wait upon the discovery and elucidation of the properties of the more spectacular dorsal lip of the blastopore. The work on the organizer led to its attempted analysis from the chemical viewpoint. Following the announcement in 1932 (Bautzmann, Holtfreter, Spemann, and Mangold, 1932) of induction effects obtained from organizers killed in various ways, a field more comprehensively covered later by Holtfreter (1933, 1934*a*), came the simultaneous attempts by Spemann, Fischer, and Wehmeier (1933) and by Waddington, Needham, and Needham (1933) to isolate the active principle involved in induction from dead materials and to determine its chemical nature. The inductive potency of variously prepared extracts was successfully demonstrated (reviewed by Weiss, 1935), but the conclusion that a particular substance is the inductive principle is at present controversial.

The work reported in the present paper was originally undertaken in the attempt to apply to the lens problem the methods used in the study of the chemical nature of the organizer. The problem under consideration was the effect of dead optic vesicle and of extracts of dead vesicle upon living presumptive epidermis and upon body ectoderm. During the period in which I have been engaged in the problem, Lopashov (1935, 1936) reported results obtained from a similar approach. He had adopted as a working hypothesis the idea that specific substances are concentrated in the tissue of an organ at the time of its origin. These specific substances would consequently be excreted in abundance by a dead rudiment and should induce in the ectoderm of a gastrula chiefly rudiments like the dead inductor. His experiments consisted of transplanting dead optic vesicles from *Amblystoma mexicanum* and *Triton taeniatus* into the blastocoel of early gastrula stages of *T. taeniatus*. Explants of dead optic vesicles enveloped in a piece of the prospective ectoderm of the same early gastrula stages were also used. Transplantations of living optic vesicles at the same stage served as control experiments. In both cases from one to seven optic vesicles were transplanted.

The dead optic vesicles in both whole embryos and in explants induced rudiments consisting chiefly of eye tissue. A quantitative effect was evident in that one dead optic vesicle gave no induction, whereas 2–7 dead vesicles always induced eye rudiments. Seven inductions were obtained in explants and seven in whole embryos. The eyes induced in the explants were irregular, having the appearance of hollowed outgrowths of retinal tissue. In almost every case they were arranged symmetrically on both sides of a small brain. In one explant two small lenses appeared attached to the ectoderm. In whole embryos the inductions are said to resemble regular eyes well isolated from the brain rudiment. Each eye had induced a lens in the overlying ectoderm. When 7 dead eye vesicles were transplanted simultaneously, three eyes were induced in the explant. Living optic vesicles induced lenses from gastrula ectoderm, but no brain or eye rudiments.

A successful demonstration of the specific effect of a dead inductor would be important in its consequences if it could be generally extended, although it would conflict with previous experience in a far from thoroughly explored field. A very brief summary may serve to orient this aspect of the problem in the general field of amphibian organization. In the first place, the cumulative evidence of today indicates that the quality of organized inductions in host embryos is due to specific influences imposed by living grafts on their surroundings. The "head" and "trunk organizers" of Spemann (1931) and Hall (1932), the specific effect of different levels of the archenteron roof when transplanted into indifferent ectoderm (Mangold, 1933*a*), and the specificity of the inductions brought about by regional fragments of the medullary plate (Mangold, 1933*b*) are cases in point. In the characteristic cases of this type of induction there is no question of stimulation or activation of latent responses in the host tissue.

When dead organizers or extracts of dead organizers or other tissues were introduced into host embryos, the quality of the organized inductions obtained was not determined by the inductor. Weiss (1935) commented: "This consistent lack of specificity in the effects of dead organizers is a general observation, apparently one of the very few features which the bewildering number of inductors, examined so far, have consistently in common." He believed that the organizing principle in these experiments with dead inductors in host embryos was not provided by the "inductor" but by the reacting ectoderm, or, in other words, "by activated fields of the host." The strongest argument for this view was the fact that the quality of the inductions corresponded approximately to their location in the host germ. The fact that living organizers produced specific effects while dead organizers or tissue did not is generally acknowledged; it is in the interpretation of this fact, as seen in Weiss's emphasis on the role of the host fields, that differences arise. Holtfreter (1934*a*) attempted to account for the diversity of effects by postulating a corresponding variety of inductive substances.

The relation of the type of induction obtained in isolated fragments of prospective ectoderm under the influence of dead inductors (Holtfreter, 1933, 1934; Lopashov, 1936) to the foregoing two types of induction is at present obscured by conflicting results. Holtfreter found that in a neutral medium such explants merely proliferate and form epidermis. If brought into contact with a dead inductor they differentiate into neutral tissue, tubes, and vesicles of neural character, but without organization into recognizable parts. Under one condition, when coagulated embryonic chick extract was used as inductor, development of chorda and somites was observed, as in Bautzmann's (1929) experiments with interplantation of ectodermal explants into the eye orbit. Lopashov's results, as described above, indicated a specific inductive effect upon explanted prospective ectoderm.

The present paper reports results which are interpreted as confirming, on the whole, the idea of specific inductive effect on the part of a dead inductor in explanted prospective ectoderm. The influence of the dead optic vesicle on the differentiation of body ectoderm from the neurula stage and on the prospective ectoderm of the early gastrula was studied in the embryo of *Amblystoma punctatum.*

MATERIAL AND METHODS

The eggs of *A. punctatum* were used exclusively in these experiments. All operations were done under the binocular dissecting microscope with the aid of fine steel needles. The dead material required was prepared by removing the optic vesicles cleanly from early tail-bud stages in which the overlying ectoderm was not yet adherent to the vesicle. With a micro-pipette the vesicles were dropped into 95 per cent alcohol and later dried by placing the dish at 60° C. overnight. This procedure produced a firm, discrete mass of dead material representing one optic vesicle which could easily be picked up as a whole by a needle. Vital staining to identify the dead vesicle was found to be unnecessary; the alcohol fixation of the dead vesicle contrasted in the later staining of the explant fixed with formalin saturated with mercuric chloride. The sharp outline of the dead vesicle in stained sections of the explant made it easily recognizable.

An extract of dead optic vesicle was prepared as follows: optic vesicles were removed from some 25–30 embryos of the usual stage, ground up with anhydrous sodium sulphate and placed in a desiccator until dry. This material was extracted with ether for 48 hours at 30° C. and then filtered. Droplets of oily sediment remained in the filtrate after the ether was evaporated off. This was dissolved in 1 cc. of equal parts of acetone and ether; a small quantity of warm egg albumin and one drop of nile-blue sulphate were added and the mixture coagulated over hot water. Gentle heating was continued until no smell of either ether or acetone was detected. Suitably small pieces of this coagulum were placed between pieces of body ectoderm.

The ectoderm used to test the inductive potency of the dead vesicle was taken from U-shaped blastopore and yolk-plug stages (Harrison's Stage 11 and 12, respectively); body ectoderm from neurulae was used to test the extract preparations and the dead vesicle. A technique for the maintenance of sterility was followed. In order to provide as neutral an environment for the vesicle as possible, free of possible correlative factors generated at a distance and spread over the surrounding field where they might complicate the effects induced by the test object, the explantation method described by Holtfreter (1931, 1933) was adopted and used exclusively in these experiments. A piece of presumptive belly epidermis was removed from the embryo and freed of yolk if necessary. If a single dead vesicle was to be placed in the explant, this piece of ectoderm was cut in two and the vesicle put on one piece and covered by the other, like the meat in a sandwich. If the enveloping ectoderm was left in a single piece and turned like an omelette to cover the vesicle, extrusion of the latter usually occurred. For two or more dead vesicles two pieces of ectoderm from two embryos were necessary to cover the dead material. Fusion of the edges took place in about an hour, and the explant was then transferred to a covered, flat Stender dish, half full of sterile salt solution (Holtfreter, 1931, 1933). The explant rested on a piece of lens paper.

After seven days of cultivation the explants were killed and stained *in toto* with alum carmine-picric acid solution. Since the diameter of the average explant was about 1 mm., early staining facilitated its handling through vials of dioxan and paraffin.

EXPERIMENTAL

EXPLANTS OF ISOLATED PRESUMPTIVE EPIDERMIS

Explants of presumptive epidermis alone, which had not been in contact with either living or dead optic vesicle, constituted one type of control experiment. A series of 25 such explants was made of presumptive belly epidermis from early gastrulae and, after 7 days' cultivation in the usual sterile salt solution, killed for sectioning. The 16 explants sectioned showed only epidermis—no nervous tissue of any kind. The often irregularly arranged masses consisted of isodiametric epidermal cells among which many cysts of various sizes appeared to indicate secretory activity on the part of the epidermal cells under cultivation (Fig. 4). Similar explants of presumptive epidermis from neurulae also yielded typical epidermal balls. Holtfreter (1933) pointed out that when the ectoderm fastened itself to the glass bottom of the dish instead of rounding up into a ball, as it does when kept away from the bottom, a special case appeared. Then its surface was smoother and the cell mass more compact. In either case, however, the end product of its differentiation was pure, irregularly arranged epidermis.

EXPLANTS CONTAINING EXTRACTS OF DEAD VESICLES

Small masses of coagulated egg albumin which had been impregnated with the ether and acetone extract of a quantity of dead optic vesicles were placed in explanted fragments of the body ectoderm of neurulae. The results were uniformly negative; the epidermis showed no signs of a lens reaction. In some explants very extensive cavities appeared and the surface epidermis was markedly thickened over a large area. No localized area of proliferation was observed and no trace of induced neural structures. Dead optic vesicles in explanted body ectoderm of this stage likewise induced neither a lens reaction nor structures of any kind.

EXPLANTS CONTAINING TWO OR MORE DEAD OPTIC VESICLES

The effect of increasing the number of dead optic vesicles in each explant was studied in explants of prospective ectoderm; a series with two dead vesicles to an explant will be considered first. Three explants made with ectoderm from early gastrulae of Stage 11 died within 3 days; but of 12 explants made with ectoderm from yolk-plug stages, 4 appeared to be alive on the seventh day and were sectioned. One was so disintegrated as to be useless; another may have been dead when fixed but its interior was filled with neuroid masses. Since neither of the dead optic vesicles was identified, their extrusion may have played a part in the survival of the explant. The same picture of necrotic conditions was to be seen in the third explant and the same absence of dead optic vesicles, but the induced masses were here organized into a thick-walled neural structure surrounding a central canal. The fourth explant contained one of its original two dead vesicles in a partially extruded condition and also a large neuroid mass of no discernible organization.

Six dead optic vesicles were placed in each of 2 explants of presumptive epidermis from early gastrulae (Stage 11). The enveloping ectoderm had completely disintegrated by 48 hours.

A series of 8 explants, each of which contained 5 dead optic vesicles, was prepared from ectoderm of yolk-plug gastrulae (Stage 12). These explants went to pieces in 5 days; the optic vesicles were often recognizable as compact bodies lying in the cellular debris of dead ectoderm. Other vesicles had been extruded from the explants as early as the second day.

The fate of these 25 different explants appeared to warrant the conclusion that the early gastrula ectoderm of *A. punctatum* could not survive close contact with large amounts of dead material. The lethal effect of 5 or 6 dead optic vesicles on responsive ectoderm was made evident before any inductive effect could be studied. The mortality of explants with only 2 dead vesicles was impracticably high, but the inductive effect observed in the 3 explants where complete or partial extrusion of the dead material had apparently allowed the survival of the piece suggested that 1 dead optic vesicle might be tolerated in the explant.

EXPLANTS CONTAINING ONE DEAD OPTIC VESICLE

The prospective ectoderm of explants containing 1 dead optic vesicle gave no indication of being adversely affected by this amount of dead material. It responded to the stimulus of the vesicle with some form of neural differentiation in slightly over half the total number of surviving explants. The specificity of this response is, however, the point in question at the present time, and it may be indicated in advance of detailed consideration that of 17 explants showing varying degrees of neural differentiation, only 3 can be said with any likelihood to have responded with the differentiation of an eye rudiment.

Table 1 summarizes the effects induced by 1 dead optic vesicle in explants made of prospective ectoderm from 2 early stages, the U-shaped blastopore and the yolk-plug stage (Harrison's Stages 11 and 12, respectively). In 3 instances the dead vesicle was not found within the explant, nor in its vicinity. Presumably it had been extruded soon after explantation; the ectoderm differentiated into typical pure epidermis. In 13 explants the dead vesicle was identified within the explant and yet the epidermal character of the ectoderm was unchanged. The resulting appearance of the explant was that of a typical epidermal ball. In 5 of these 13 explants the dead vesicle was partially extruded, but the other 8 in which it was completely covered by the epidermal envelope were the same in appearance. Moreover, neural differentiation was observed in other explants where partial extrusion had occurred. Actual contact between the ectoderm and the dead material was apparently not a factor in induction; in many explants the dead vesicle was surrounded on all sides by masses of cells in the closest contact, while in others it lay within a considerable cavity.

In all the remaining explants the dead optic vesicle was identified within the explant and some degree of neural differentiation had resulted. In 5 cases the neural character of the differentiation was evident, but the degree of organization was so slight that the resulting areas could only be called neuroid in character. The change in the nuclei of the cells was particularly marked and an elongation of cell and nucleus was distinct. Tightly packed groups and masses of cells showed indistinct radial arrangement in contrast to the haphazard scattering of cells in other regions of the explant. Sharply defined crevices appeared bordered by orderly rows of elongated cells, but recognizable neural formations were lacking.

In 9 explants the evident neural character of the differentiation was largely confined to formation with sharply defined boundaries. The structures formed were bizarre and incomplete, as, for example, two well-formed neural tubes which fused into a large neural mass with no central cavity. In general, the amount of tissue involved was small compared with the size of the explant as a whole, but in one case a mass of fused neural tubes almost filled the interior of the explant, while at the other extreme of size was a small neural tube which lay in a large cavity at one end of the explant. In all cases the induced structures were neural in character, either short neural tubes, irregular plates and folds, or unrecognizable brain masses. Organization in the sense of an orderly arrangement of parts was lacking. Nothing of mesodermal or entodermal origin was seen; no structure resembling notochord, gut, or somites was observed.

TABLE 1

THE CONDITION OF EXPLANTS WITH 1 DEAD OPTIC VESICLE AFTER 7 DAYS' CULTIVATION, AS SEEN IN SECTION

	Stage 11	Stage 12
Dead optic vesicle wholly extruded; epidermal ball with no indication of neural differentiation	1	2
Dead optic vesicle identified in explant; epidermal ball with no indication of neural differentiation	6	7
Dead optic vesicle identified in explant; differentiation consists of neuroid masses	4	1
Dead optic vesicle identified in explant; differentiation consists of neural tubes and/or parts of brain	8	1
Dead optic vesicle identified in explant; differentiation consists of brain masses and optic rudiments	0	3
Explants lost in transfer or dead by seven days	3	5
Total	22	19

Three explants, which, in the sense of representing definitely neural rudiments, might be included among the foregoing class are dealt with separately because they presented one feature either missing or extremely obscure in the other explants. This feature was a structure connected with a brain mass which resembled an optic vesicle in general configuration and cellular arrangement.

Explant 112 presents the best example. The induced neural masses lie within a large cavity bounded on three sides by a thin layer of epidermis (Fig. 3). The dead optic vesicle lies at the opposite end of the explant, a considerable distance from the induced mass and does not appear in the section shown. Figure 1 represents an attempt to show graphically the aspect of the entire induced mass in 3 dimensions. Each figure represents roughly one-half the whole mass, although a section slightly larger than the usual interval of 32 μ is unrepresented in the middle of the mass and is responsible for the difference in profiles. The stippled area represents the extent of the hollow interior at the level of the last section in each half. The large round mass of closely packed cells at no time possessed a cavity and shows no connection with the larger mass with which it is in contact at some levels. The structure resembling the optic cup appears at one end of the larger mass from which it is separated by a deep constriction. The two cavities seen in the photomicrograph are continuous at some levels. Two small round objects composed of radially directed wedge-shaped cells resemble early stages of lens vesicles; one of them is apparently continuous with the large neural mass. For comparison with noninduced brain rudiments grown in explant, a secton through a control explant which shows a lens in process of formation is included (Fig. 5). This explant consisted of a section of the anterior end of one neural fold which had not completely fused with the opposite fold, enveloped in late gastrula ectoderm. The brain rudiment was alive when explanted and included the rudiments of optic vesicle of that side. The photomicrograph (Fig. 5) shows the condition of the rudiment at the end of 3 days' cultivation, a period during which a lens vesicle has formed in the enveloping ectoderm. The lens vesicle is the only induced structure produced in the explant. In the absence of lens fibers at this stage of development, the identification of such lentoid structures as seen in Figure 3, with the lens in the control, must be tentative.

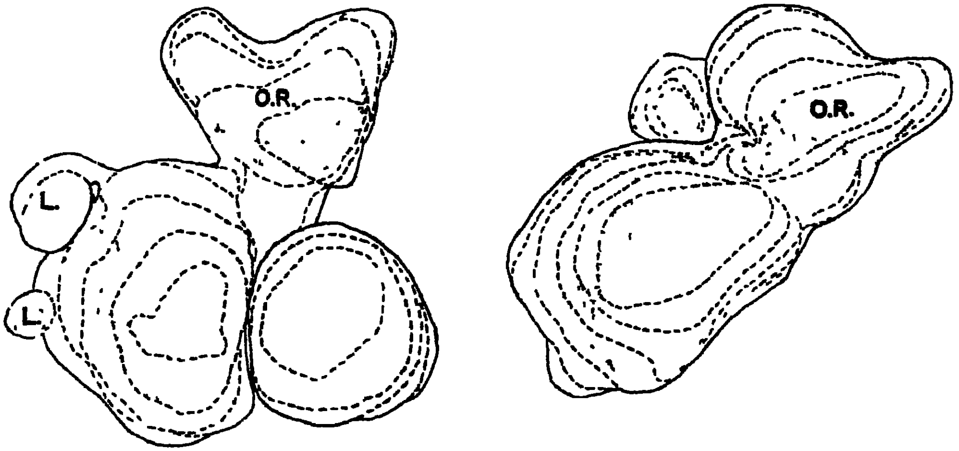


FIG 1.—Graphic representation of the two halves of Explant 112, a section of which is shown in Figure 3. The dotted lines indicate the outlines of the structures as seen in every fourth section (32 μ) The stippled area represents the extent of the hollow interior at the final level shown; a narrow middle section is unrepresented and accounts for the difference in profiles.

Explant 72, a section through which is seen in Figure 2, also presents a mass of induced brain parts and a structure like an optic cup. The dead optic vesicle does not appear in the section. The large neural tubelike structure is more intimately connected with the other hollow rudiment at other levels but the solid mass below has no connection with other structures. No lentoid structures appear in any section. Explant 73 was not in the best condition when fixed, but the sections do show a central brain mass from either side of which protrudes a structure interpreted as an optic vesicle. Associated with one of these induced optic vesicles is a structure which may be a lentoid.

Another series of 16 explants consisting of 1 dead optic vesicle in yolk-plug stage ectoderm but not included in Table 1 may be briefly mentioned. Four explants were killed at the end of 3, 4, 5, and 9 days in the attempt to follow the successive stages in the development of the induced structures. Of the 4 killed after 3 days' cultivation, 2 had extruded the optic vesicle and showed no organization. In the other 2 the cells surrounding the dead vesicle were arranged in concentric whorls and, for a short distance, in orderly rows facing a considerable cavity, otherwise the appearance was much like a typical epidermal ball. Among the explants killed after 4 days was 1 showing definite neural masses, although the dead vesicle had been extruded, this was also true in another killed after 5 days. Another of the same age showed the condition of concentric whorls of closely packed cells seen in a less marked form in 3-day explants. If this condition was preliminary to definitive organization, there was nothing to account for its later appearance here when a 4-day explant had exhibited definite neural masses. The 9-day explants showed only masses of neuroid tissue.

Although the low percentage of inductions defeated the purpose of this series, it is evident that morphological changes which may result in neural structures or which, at the very least, are not epidermal in character, are under way after 3 days' cultivation of the explant. It cannot be concluded from the evidence at hand that the same processes, if continued 24 hours, would produce the definitely neural structures found in the 4-day explant. That the neural induction appeared at all in an explant from which the dead vesicle had been extruded indicates that the continued presence of the latter is unnecessary for induction and suggests that a minimum period of contact might be established.

DISCUSSION

The present status of knowledge concerning the effect of dead inductors in whole embryos and in explanted indifferent ectoderm has been adequately indicated for present purposes in the introductory section. Wide generalizations as to the theoretical implications of qualitatively specific induction seem premature and hazardous in view of the few and conflicting reports on this aspect of the problem of amphibian organization. This discussion will be confined to the experimental results presented above and their relation to similar work.

The positive evidence adduced in this paper for the existence of a specific inductive effect by a dead inductor consists in the induced formation of optic rudiments in isolated fragments of presumptive epidermis which enveloped a dead optic vesicle. The fact that this specific induction occurred in only 3 explants is of secondary importance; it would appear easier to explain why the effect failed to appear than why it did. Some importance can also be attached to a sort of negative evidence, namely, that dead eye rudiment induced eye rudiment and not other neural structures, such as nasal placode and ear vesicle. Further experiments using these rudiments as inductors would appear to constitute valuable and critical controls. In a brief communication Lopashov (1937) stated that he had obtained inductions of nasal placode and ear vesicle from like dead rudiments.

A possible explanation of the gradation of inductive effects seen in Table 1 may be found by postulating a difference in intensity or amount of a hypothetical inductive substance present in the dead eye rudiments. Some explants showed no signs of response to the enveloped dead inductor and differentiated as epidermis; others showed sufficient change in the neural direction to be called neuroid; in still others the induction was definitely neural in character and formed recognizable neural structures; but in only 3 explants were eye rudiments produced in addition to the induced neural structures. The gradation in effect may reflect subminimal and increasing amounts of inductive substance. Holtfreter's results emphasize that the inductive effect is not of the all-or-none character. Dead inductors and tissues from different sources showed definite gradations in the effects produced. The materials classified as "strong" by him induced a wide diversity of derivatives of all 3 germ layers; the "weak" induced ectodermal derivatives only.

A point of difference between Lopashov's results and my own was the quantity of dead optic-vesicle material necessary for induction. He obtained no induction in either whole embryos or explants with only 1 dead vesicle: at least 2 were necessary for the effect, while with 7 dead vesicles eyes were induced. My experience with *A. punctatum* was that quantities of more than 1 dead vesicle were lethal. This susceptibility may, of course, be a specific difference between the animals used. The importance of the quantity of inductor necessary for induction in *A. mexicanum* and *Triton* gives support to the suggested quantitative explanation of my results.

The part played by the responding ectoderm in these experiments was critically delimited by employing the explanation method. The fragments of prospective ectoderm, isolated in a neutral medium, would appear to provide an ideal proving-ground for inductive effects. They are isolated at the very beginning of gastrulation, have apparently unrestricted potencies (Holtfreter, 1933, 1934*b*), and, in contrast with the same areas *in situ* during development of the whole embryo, are free of the criticism that the spread of various overlapping "fields" of the host has influenced or dictated the nature of the inductions. The salient point of a specific inductive effect is that the characteristic quality of the induced rudiment is imposed upon the responding tissue by the inductor. There can be no question of mere activation or stimulation of a latent response which is evoked by a diversity of inductors. Although I have not yet tried the effect of other dead inductors of a narrowly circumscribed nature, I believe that the results of my experiments indicate that the influence of the dead optic vesicle is imposed on the indifferent ectoderm end so constitutes true induction rather than activation.

SUMMARY

1. The influence of dead optic vesicles and of extracts of dead vesicles upon explants of prospective ectoderm of early gastrulae and of body ectoderm from late neurulae of *A. punctatum* was studied in these experiments.

2. An ether extract of dead optic vesicles induced neither lens nor other structures in explanted body ectoderm of neurulae.

3. Explanted fragments of prospective ectoderm differentiated as epidermis.

4. Explants of prospective ectoderm containing 2 or more dead optic vesicles were killed by this amount of dead material.

5. Explants of prospective ectoderm containing 1 dead optic vesicle showed inductions of definite neural character after 7 days' cultivation. Pattern and organization were lacking in the induced masses; entodermal and mesodermal derivatives were not observed. In 3 such explants were found structures resembling optic rudiments connected with a brain mass.

6. These optic rudiments induced by dead optic vesicle are adduced as evidence of a specific inductive effect of the dead inductor upon explanted presumptive epidermis.

7. The relation of the results of these experiments to other similar work is discussed.

LITERATURE CITED

BAUTZMANN, H. 1929. Ueber bedeutungsfremde Selbstdifferenzierung aus Teilstücken des Amphibienkeimes. Naturwiss., 17:818.

BAUTZMANN, HOLTFRETER, SPEMANN, and MANGOLD. 1932. Versuche zur Analyze der Induktionsmittel in der Embryonalentwicklung. Naturwiss., 20:971.

HALL, E. K. 1932. Die Wirkung regionaler Verschiedenheiten im Organisationszentrum. Roux's Arch., 127:573.

HOLTFRETER, J. 1931. Ueber die Aufzucht isolierter Teile des Amphibienkeimes. II. Züchtung von Keimen und Keimteilen in Salzlösung. Roux's Arch., 124:404.

———. 1933. Nachweis der Induktionsfahigkeit abgetöteter Keimteile. Isolations- und Transplantationsversuche. *Ibid* 128:584.

———. 1934*a*. Der Einfluss thermischer, mechanischer, und chemischer Eingriffe auf die Induzierfähigkeit von Triton-Keimteile. *Ibid.*, 132:225.

1934*b*. Ueber die Verbreitung induzierender Substanzen und ihre Leistungen in Triton-Keim. *Ibid.*, p. 307.

LEWIS, W. H. 1904. Experimental studies on the development of the eye in Amphibia. I. On the origin of the lens. *Rana palustris*. Amer. Jour. Anat., 3:505.

LOPASHOV, G. V. 1935. Induction of the eye by a specific substance in the Amphibia. Nature, 136:835.

———. 1936. Eye inducing substances (English summary). Biol. Zhur. 5(3):463.

———. 1937. Ueber die Spezifität induktiver Einflüsse. Compt. rend. (Doklady) acad. sci. USSR, 15:286.

MANGOLD, O. 1933*a*. Ueber die Induktionsfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. Naturwiss., 21:761.

———. 1933*b*. Isolationsversuche zur Analyze der Entwicklung bestimmter Kopforgane. *Ibid.*, p. 394.

SPEMANN, H. 1931. Ueber den Anteil von Implantat und Wirtskeim an der Orientierung und Beschaffenheit der induzierten Embryonalanlagen. Roux's Arch., 123:389.

SPEMANN, FISCHER, and WEHMEIER. 1933. Fortgesetzte Versuche zur Analyze der Indukionsmittel in der Embryonalentwicklung. Naturwiss., 21:505.

WADDINGTON, NEEDHAM, and NEEDHAM. 1933. Physico-chemical experiments on the amphibian organisation centre. Nature, 132:239.

WEISS, P. 1935. The so-called organizer and the problem of organization in amphibian development. Phys. Rev. 15:639.

PLATE I

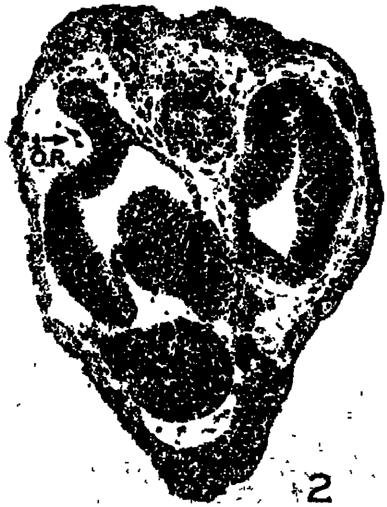
FIG. 2.—Photomicrograph of a section of Explant 72 (× 90) showing neural structures induced by one dead optic vesicle. *O.R.* = optic rudiment.

FIG. 3.—Photomicrograph of a section of Explant 112 (× 90) showing neural structures induced by one dead optic vesicle. *O.R.* = optic rudiment; *L.* = lentoid.

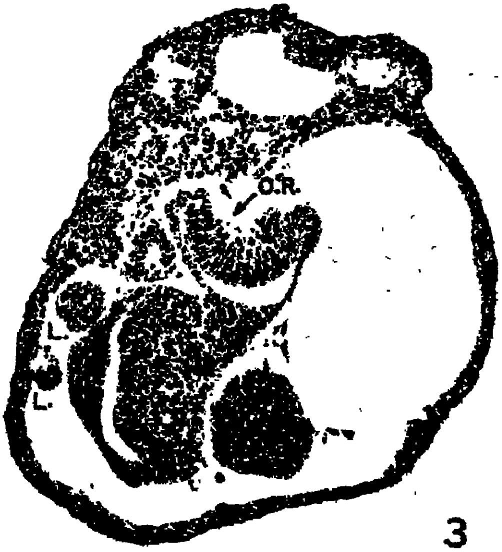
FIG. 4.—Photomicrograph of a section of Explant 87 (× 90) showing epidermal appearance of explanted presumptive epidermis after 7 days' cultivation.

FIG. 5.—Photomicrograph of a section of Explant D 25. Anterior section of a neural fold explanted in the living state after 3 days' cultivation. An optic rudiment has induced a lens vesicle (*L.*) in the ectoderm.

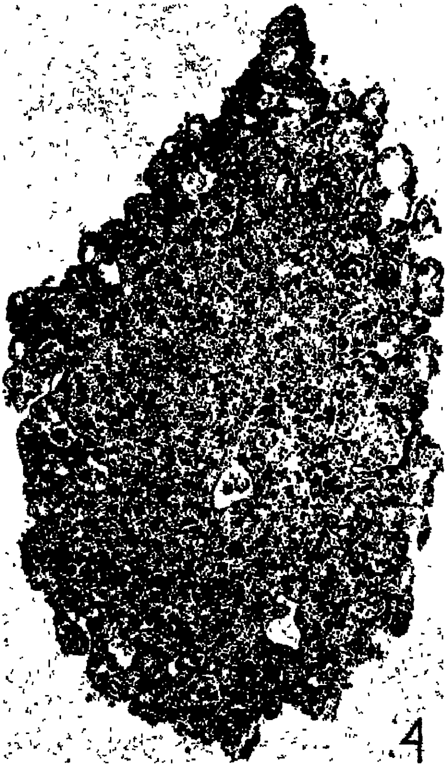
PLATE I



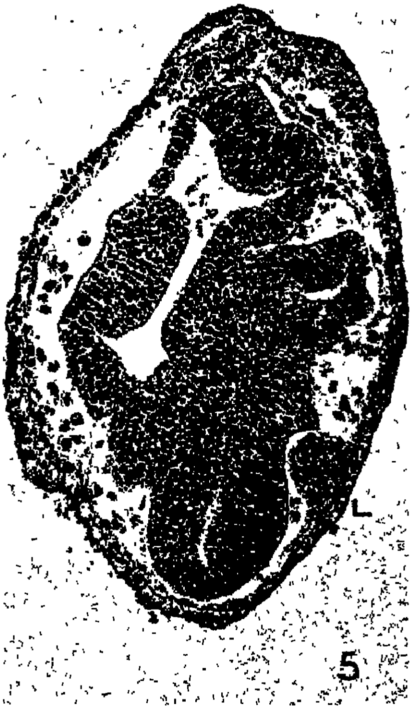
2



3



4



5

OXYGEN AS A CONTROLLING FACTOR IN THE REGENERATION OF TUBULARIA

(Four figures)

L. G. BARTH
Department of Zoology, Columbia University and the Marine Biological Laboratory
Woods Hole, Massachusetts

IT WAS suggested by Morgan (1903) that the inhibition of regeneration when the cut end of *Tubularia* was placed in sand was due to lack of oxygen. Child (1927, 1928) has shown that small pieces of *Corymorpha* in contact with the bottom of a dish develop hydranths from the free surface and stolons from the surface in contact with the glass. He suggests that the differential exposure of the surfaces of the fragment results in a difference in respiratory exchange which may determine the new polarity. There has been no attempt to study quantitatively the relation between oxygen tension and regeneration in *Tubularia* or *Corymorpha.* This paper will discuss, first, some qualitative observations on regeneration of ends of stems of *Tubularia* under conditions of reduced oxygen supply, and, second, a quantitative study of the size of the hydranth, time for regeneration, and rate of regeneration at various oxygen tensions. The experiments were carried out with the very able assistance of Mr. John Moore, who determined the oxygen tensions. The work was supported by a grant from the Rockefeller Foundation.

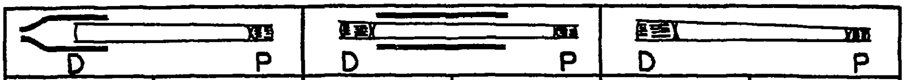
METHODS

The methods used in measuring rate of regeneration are similar to those used previously (Barth, 1938). After selection of the stems, random samples are taken for each lot and measurements of the diameter of the stem and length of the primordium of the hydranth are made when the constriction appears between the primordia and the rest of the stem. The time in hours required for the appearance of this constriction is termed t_r. The length of the primordia in micra divided by the time in hours is used as a measure of rate of regeneration in these experiments. The rate of regeneration is then measured in micra-hours or regenerative units which we shall designate R.U. For the work on oxygen tension the stems were placed in sea water at the tension to be examined, in flasks of 1,000-cc. capacity and the surface of the sea water covered with a thick layer of nujol. Siphons for taking of samples were inserted through the oil layer. With 20 stems in this large volume of water the O_2 tension did not vary over the period of the experiment. The oxygen tension was determined by Winkler's methods; usually the average of three samples taken at intervals was used.

PRELIMINARY STUDIES

In some of the studies on regeneration of cut stems of *Tubularia* it was found that when the tissue was pinched off for a distance of a few millimeters from the cut end of the perisarc no regeneration occurred. It appeared then that the tissue must be at the opening of the perisarc in order to differentiate into a hydranth. This inhibition of regeneration when the tissue is not at the opening of the perisarc might be due to lack of an adequate oxygen supply, as the diffusion rate in small capillaries such as the tube formed by the perisarc is very slow. The perisarc averages about 0.5 mm. in diameter. On the other hand, waste products might accumulate for the same reason.

Further experiments, in which the rate of regeneration was measured with the distal end of stems placed in glass capillaries about 1 mm. in diameter, showed that complete inhibition of distal regeneration occurred, with the further result that the proximal ends regenerated more rapidly (Figs. 1, *A*, *B*, *C*). The results are clear. There is no regeneration at the end which is capped with a glass capillary, while when the capillary is slipped over the stem, leaving the ends free, almost the normal rate of regeneration is found—32.1 R.U. as compared with 38 R.U. at the distal end and 4 R.U. compared with 2.7 R.U. at the proximal end. The stems used for these experiments were short, 10 mm., and thus there was almost complete dominance of the distal end over the proximal end in *B* and *C*. However in *A*, where regeneration of the distal end was prevented by the glass cap, the rate of regeneration of the proximal end rose to 21.3 R.U.



	A		B		C	
	D	P	D	P	D	P
L	0	912	1088	156	1244	100
r	0	240	242	200	244	340
t_1	-	42.8	33.9	39.0	32.8	37.0
R	0	21.3	32.1	4.0	38.0	2.7

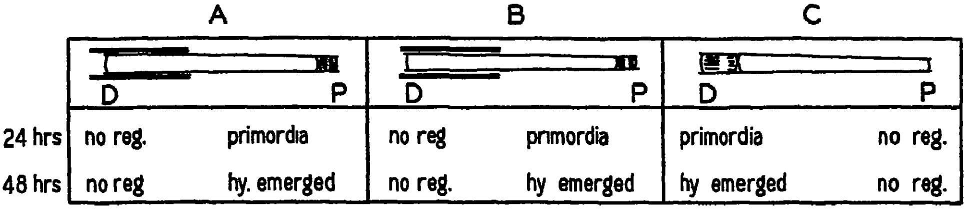
FIG. 1.—Inhibition of regeneration by covering the cut end of *Tubularia* with a glass tube. Stems 10 mm. long; 20 stems in each group, *A*, *B*, and *C*. L=length of primordium in micra; r=radius of cross-section in micra; t_1=time in hours from cutting to the formation of the primordium; R=rate of regeneration as measured by L/t_1. Group *A*. the distal end is inserted in a glass tube; Group *B*, the middle part of the stem is covered by the ends are free; Group *C* serves as untreated control; D=distal end of the stem; P=proximal end of the stem.

Examination of the distal ends within the tubes showed the coenosarc disintegrating in some cases, leaving this region of the perisarc filled with a mass of unorganized cells and cell debris. In other cases the distal end simply closed after cutting and showed no further signs of either disintegration or differentiation.

The next experiment shows that even when the glass tube is completely open at the end regeneration is inhibited. In this case the cut end was about 0.5 mm. from the opening of the glass tube (Fig. 2, *A*), but no differentiation took place. As is expected, inhibition of the distal end causes the proximal end to regenerate. These results were surprising, and the experiment was repeated with another group (Fig. 2, *B*) in which the distal ends of stems were placed in glass tubes with the cut ends flush with the ends of the tubes. After 24 hours there was no regeneration at the distal end in the tubes while the proximal free ends were regenerating. The controls (Fig. 2, *C*) showed the distal end to be completely dominant in these stems. The results show clearly that covering the end of the stem with an open glass tube inhibits regeneration. Further experiments, using a porcelain plate with small perforations, gave the same effect when the cut ends of stems were placed in the holes.

THE EFFECT OF VARYING OXYGEN TENSION UPON THE REGENERATION OF CUT STEMS

The preliminary studies indicate that reducing the oxygen supply of the cut end by means of glass tubes inhibits regeneration. In the following experiments sea water was made up at various oxygen tensions and twenty cut stems treated. The size of the developing hydranth as measured by the length of the primordium, *L*, the time for the formation of the primordium, t_1, and the time for emergence of the hydranth, t_2, were observed. The stems used were 5–7 mm. long so that dominance of the distal end was almost complete and few or no proximal hydranths developed at normal oxygen tensions. Since the stems were in sealed containers, they had to be removed for measurement. They were taken out when, by gross inspection, about 50 per cent showed primordia. After removal the stems were kept at the normal O_2 tension of sea water in open dishes. This procedure meant that the stems at higher O_2 tensions were treated for a shorter time than those at lower O_2 tensions because of the difference in rate of regeneration.



	A: D	A: P	B: D	B: P	C: D	C: P
24 hrs	no reg.	primordia	no reg	primordia	primordia	no reg.
48 hrs	no reg	hy. emerged	no reg.	hy emerged	hy emerged	no reg.

FIG. 2.—Inhibition of regeneration of cut ends of *Tubularia* in glass tubes. *A* = distal cut end about 0.5 mm. from opening of tube; *B* = distal end flush with opening; *C* = control. Stems 10 mm. in length; 20 stems in each group.

Many preliminary experiments were carried out, but the last three experiments of the series will be used chiefly for this paper. The data from these are included in Table 1. Here the oxygen tension is recorded, the average lengths in micra, the average time in hours, and finally the average rate of regeneration of 20 stems is calculated as $R = L/t_1 =$ R.U. When a stem completely fails to regenerate, it is not included in the calculations. When a stem fails to regenerate during the experimental period but regenerates later, it is considered to be inhibited and it is included in the calculations. The length of the primordium of these latter will be 0, since it does not appear during the experiments, and the rate of regeneration, 0. This treatment of the data is necessary, as in low O_2 tensions the weaker stems fail to regenerate, while at higher O_2 tensions they are able to do so. Naturally in trying to measure the effect of O_2 on regeneration these weaker stems must also be included.

As seen from Table 1, with increase in O_2 tensions there are an increase in size of the primordium of the hydranth, a decrease in the time for its formation, and an increase in the rate of regeneration which is a function of these two variables. The magnitude of the effect is great, so that the rate of regeneration of stems from the same source ranges from 22 R.U. at 1.3 cc. O_2/l to 67.1 R.U. at 8.6 cc. O_2/l.

The data from Experiment 9 in which the oxygen tension of laboratory sea water was 4.7 cc./l are plotted in Figure 3. All the Experiments 1–8 agree with Figure 3 in showing that the greatest effect of O_2 tension is between 3 cc. and 5 cc. of O_2 per liter. This fact is of great importance since this range includes the normal oxygen tension of sea water in the laboratory. Any experiments on the rate of regeneration of *Tubularia* must take into consideration this critical range of O_2 tension.

Below 3 cc. of oxygen per liter the rate of regeneration falls off less rapidly, until a tension of about 1 cc. is approached, when the rates of regeneration decline sharply. At about 0.35 cc./l regeneration is inhibited completely, while above 5 cc. the rate continues to increase, but more slowly. The relation between O_2 tension and rate of regeneration from 4 cc. to 21.6 cc./l is approximately logarithmic, as shown in Figure 4. Here the rates of regeneration of Experiments 5, 7, 8, and 9 are plotted as functions of the logarithm of the oxygen tension and show a linear relationship. The older stems are more susceptible to changes in O_2 tension since the slope of these lines is greater than the slope of plots from younger stems. Compare Experiments 5 and 7 with 8 and 9. In Experiments 8 and 9 the stems were so similar that their rates of regeneration fell on the same line.

TABLE 1

THE EFFECT OF VARYING OXYGEN TENSIONS ON THE RATE OF REGENERATION OF *Tubularia*

C = control in open dish

Experiment	O_2 Cc./Liter	L Micra	h Hours	$R = L/h$
Experiment 7: 180 stems 5–7 mm. length, young....	2.4	1,072	36.1	29.7
	3.2	1,284	28.1	45.7
	4.1	1,370	26.8	51.3
	4.8	1,365	26.3	52.0
	8.2	1,640	24.5	67.0
	11.3	1,809	24.6	71.8
	14.3	1,840	24.1	75.5
	16.5	1,846	23.7	77.0
	C	1,370	26.2	52.2
Experiment 8: 160 stems 5–7 mm. length, medium...	2.9	990	36.0	27.4
	3.1	1,050	32.5	32.2
	3.3	1,072	34.2	31.0
	3.9	1,192	32.4	36.7
	4.5	1,190	32.1	37.0
	4.9	1,350	27.8	48.5
	6.5	1,412	26.4	53.5
	7.4	1,635	24.9	65.8
Experiment 9: 160 stems 5–7 mm. length, old.......	1.3	1,170	53.3	22.0
	1.8	1,250	48.1	26.0
	2.3	1,260	46.2	27.4
	3.0	1,270	42.7	29.7
	4.1	1,250	35.6	35.2
	4.7	1,420	29.7	47.7
	6.4	1,520	26.1	58.3
	8.6	1,710	25.4	67.1

The lower limit of O_2 tension for regeneration has not been accurately determined, but at 0.35 cc./l the stems may remain indefinitely without regenerating at either end, while at 1.33 cc./l most of the stems regenerate at the distal end, giving a rate of 22 R.U. Thus there must be a rapid drop from 22 to 0.0 R.U. between 1.3 and 0.35 cc. of oxygen per liter.

The highest O_2 tension tried was 21.6 cc./l, in which the stems gave a rate of 57.8 R.U. as compared with a rate of 50 R.U. at 14.8 cc./l. The range from 10 cc./l to 20 cc./l is characterized in all experiments by a flattening of the curve (Fig. 3).

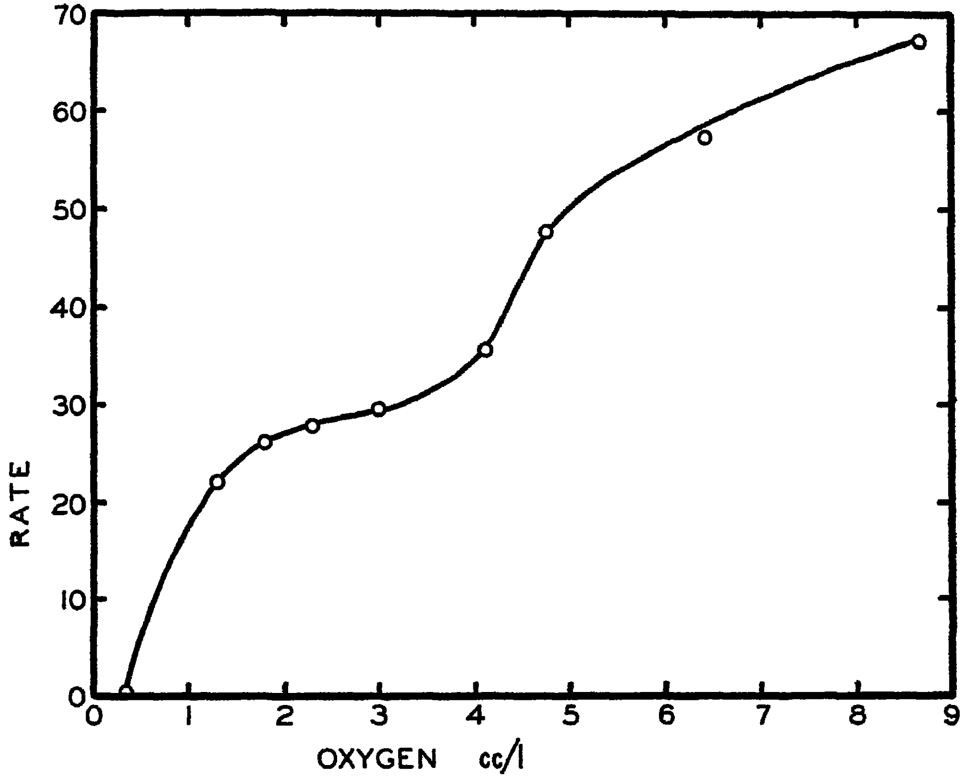


FIG. 3.—The effect of varying the oxygen tension on the rate of regeneration of *Tubularia*. Rate = L/t_1, where L = length of primordium in micra and t_1 = time in hours. Oxygen tension is recorded in cubic centimeters per liter.

The beneficial effects of high O_2 is demonstrated by the stems of Experiment 9. These stems were removed from the flasks containing O_2 at various tensions and placed in a dish of sea water for observation. The distal primordia were measured and the distal hydranths emerged, and then, as is characteristic on standing in unchanged sea water, the hydranths, after about 96 hours, dropped off and stolons grew out of the proximal ends of the stems. After 264 hours there were no hydranths present in the 160 stems, but the stems were not dead, as circulation continued. The stems were then placed in high O_2 of 14 cc./l and in 48 hours almost all the stems formed new hydranths.

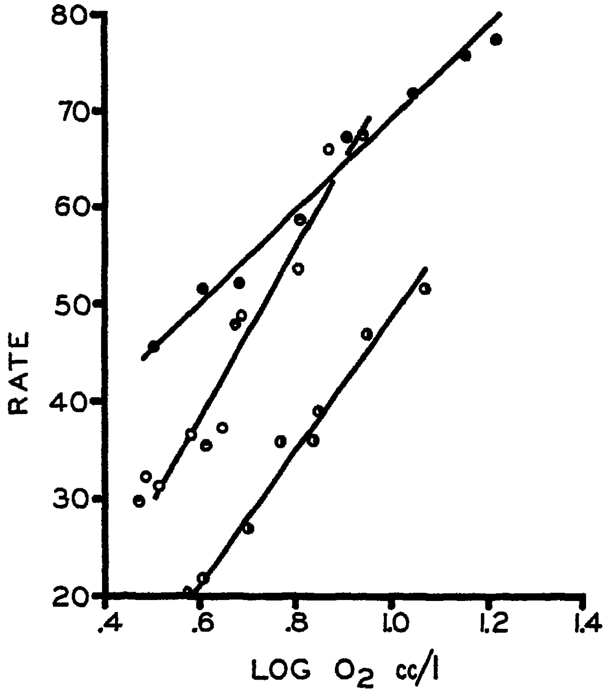


FIG. 4.—Rate of regeneration of *Tubularia* at varying oxygen tensions. Rate $= L/t_1$ in micra/hours Data from four experiments. Curve showing greatest slope is from Experiments 8 and 9 on relatively old stems. The other two curves are from Experiments 5 and 7 on relatively young stems.

TABLE 2

EFFECT OF VARYING OXYGEN TENSIONS ON THE RATE OF REGENERATION OF *Tubularia*

Experiment 2: length 15 mm.; 13 stems in each sample; treated for 40 hr., then removed to open dish for observation; L = length; t_1 = time in hours; $R = L/t_1$ = micra/hours.

	DISTAL			PROXIMAL		
O_2 Cc./Liter	L Micra	t_1 Hours	$R=L/t_1$ R.U.	L Micra	t_1 Hours	$R=L/t_1$ R.U.
1.3..........	1,080	114 0	9.1	0	....	0 0
4.76........	1,200	48.3	25.0	564	48 7	11.6
8.08	1,210	48 0	25.4	440	56 0	7.9
13.75........	1,730	40.0	43.4	720	50.5	14.3
19.1.........	1,820	40.0	46.5	980	49.4	20.0

THE EFFECT OF OXYGEN ON DOMINANCE OF THE DISTAL OVER THE PROXIMAL END

In most of the experiments the stems were chosen of such length, 5–7 mm., that almost complete dominance resulted. In one experiment 15-mm. stems were used and proximal hydranths developed in the higher O_2 tensions but not in the lower ones. Table 2 gives these results. At 1.3 cc./l the proximal ends do not regenerate, while at 19.1 cc. the rate is 20 R.U. Observation on Experiment 9 also showed that at O_2 tensions of 8.65 cc./l, 60 per cent of the proximal ends formed hydranths; 15 per cent, stolons; and 25 per cent showed no action. Lowering the O_2 tension to 4.1 cc./l gave 20 per cent hydranths, 50 per cent stolons, and 30 per cent no action. These stems, although 5–7 mm. long, were older than in other experiments and dominance was accordingly less evident.

DISCUSSION

The close dependence of rate of regeneration upon oxygen tension is extremely interesting in relation to studies on the control of polarity in *Tubularia* and other allied coelenterates. In the first place, the earlier results of Morgan (1903) on placing the end of *Tubularia* in the sand and inhibiting regeneration are clearly due to a lack of oxygen. Even in open tubes, as in my experiments, the oxygen is used up so fast that diffusion is unable to supply enough for regeneration. Second, Child's observation (1928) that the free surface of *Corymorpha* fragments always becomes the hydranth can be interpreted on the basis of higher oxygen supply. Third, the lack of regeneration when the ends of *Tubularia* are ligatured is almost certainly due to a lack of O_2, for it is clear that the perisarc is not freely permeable to O_2. This explanation is confirmed by the observation that when the perisarc cracks near a ligature the exposed tissue will start to form a hydranth, i.e., admission of O_2 from sea water allows for regeneration. Further experiments on the stimulation of regeneration by rupturing the perisarc will be reported in a separate paper. It is suggested that the stimulus for regeneration is the exposure of tissue to high oxygen. Aside from the light that these experiments throw upon the mechanism of determination of polarity, the physiological mechanism involved is of extreme theoretical importance. How does increased oxygen tension act? Does it increase O_2 consumption and so perhaps increase metabolism generally so that the processes of regeneration are hastened? Or does the increased O_2 tension simply increase the rate of diffusion of O_2 into the stem so that it penetrates more deeply and thus stimulates more of the stem to form a hydranth? This latter suggestion would account for the larger hydranths at high O_2 tensions, but not the decrease in time for regeneration. The same two possibilities exist for the explanation of the inhibition due to low O_2 tensions.

No data presented in this paper can be used to distinguish between these two possibilities. The relation between O_2 tension and O_2 consumption will have to be determined. Henze (1910) says that the O_2 consumption of two coelenterates, *Actinia* and *Anemonia*, is dependent on O_2 tension, while in two others, *Pelgia* and *Carmarina*, the O_2 consumption is independent of O_2 tension.

We have good evidence that rate of diffusion is an important factor. The experiments cited in this paper show that at the same O_2 tension the ends of *Tubularia* in open tubes will not regenerate while those free in the water will. The only explanation lies in the reduced O_2, owing to utilization by the end and failure of diffusion to supply the minimum tension required for regeneration.

SUMMARY

1. Experiments in which glass tubes were used to cover the cut end of *Tubularia* show that diffusion of O_2 at the tension of sea water, through less than a millimeter of 1-mm. glass tubing is not sufficient to supply the tissues for purposes of regeneration.

2. Varying the oxygen tension of sea water affects the size of the hydranth primordia, the time for formation of the primordia, and the rate of regeneration as measured by those two variables.

3. Increasing the oxygen tension above 4.5 cc./l increases the rate of regeneration rapidly, while decrease below 4.5 cc./l causes a sharp decline in rate. *Tubularia* therefore is existing at a very critical region of the oxygen-tension regeneration curve.

4. The lower limit for regeneration is between 0.35 and 1 cc. of oxygen per liter, at which regeneration is inhibited indefinitely but the stems are not killed. The effect is reversible, regeneration taking place when the oxygen tension is increased.

5. The rate of regeneration above 4 cc. of oxygen per liter is a linear function of the logarithm of the oxygen tension.

LITERATURE CITED

BARTH, L. G. 1938. Quantitative studies of the factors governing the rate of regeneration in *Tubularia*. Biol. Bull. 74:155.

CHILD, C. M. 1927. Modification of polarity and symmetry in *Corymorpha palma* by means of inhibiting conditions and differential exposure. Jour. Exper. Zoöl., 47:343.

———. 1928. Axial development in aggregates of dissociated cells from *Corymorpha palma*. Physiol. Zoöl., 1:419.

HENZE, M. 1910. Über den Einfluss des Sauerstoffdruchs auf den Gaswechsel einiger Meerestiere. Biochem. Zeitschr., 26:255.

MORGAN, T. H. 1903. Some factors in the regeneration of *Tubularia*. Arch. f. Entw'mech., 16:125.

DIFFERENTIAL GRADIENT FUNCTIONS IN THE FEATHER GERM[1]

(Eight figures)

RICHARD M. FRAPS
Whitman Laboratory of Experimental Zoölogy, University of Chicago[2]

LILLIE and Juhn (1932) have shown that the localization of pigmentation patterns induced by hormones in saddle and breast feathers of the Brown Leghorn male or capon requires the postulation of a purely quantitative reaction gradient in the collar of the developing germ. The concentration of hormone required to effect a given pigmentation reaction at different regions of the collar was taken as the measure of the underlying physiological gradient that determined observed threshold values. By this measure the high end of the gradient in saddle and breast feathers is at or near the ventral limit of the collar (apexes of barbs), the low end is at or near the dorsal limit of the dollar (bases of barbs). Lillie and Juhn also attempted to show that the underlying, determinative gradient in the collar was primarily a gradient in rates of cell division.

In extending the earlier experimental procedures to other tracts, patterns have been obtained which indicate that the order of pigmentation in the germ is partially or completely the reverse of the order described by Lillie and Juhn as typical in the saddle and breast germs. One might, of course, take these patterns as evidence for a reversal in direction of the underlying gradient differentials postulated for saddle and breast germs. The possible existence of localized reversals in direction of the primary collar gradient has, in fact, been recognized by Lillie (1932) and cannot be excluded in certain pattern formations (penciling, for example). But, if we think of the reaction gradient (for pigmentation) described by Lillie and Juhn as the direct concomitant of primary differentials in developmental processes (e.g., rates of cell division), we must also suppose fundamental differences in the organization of feather germs exhibiting the partially or completely reverse orders of pigmentation described below. In certain germs the high end of the reaction gradient, as measured by hormone concentration required to produce a given reaction, is at the ventral limit of the collar; in others, at the dorsal limit; and in still others the gradient has high (not necessarily equal) values at ventral and dorsal limits with minimal values at intermediate collar levels. The organization of the feather germ and the primary differentials associated with its development can scarcely differ in correspondence with these relations.

Experimentally induced pigmentation patterns in the Brown Leghorn always appear as alternatives to the immediately displaced pigmentation reaction. If we assume that the conditions tending to effect each of these alternatives represent *differential functions* of a primary developmental gradient, such as rate of cell division, the reversal of threshold differentials in different germs represents only a differing relationship between two critical but derivative processes of graded intensity within the collar limits. The primary developmental gradients in different germs (gradients in rates of cell division, differentiation, etc.) are assumed, on this view, to remain unchanged *in direction* but to differ in absolute values in feather germs differing in growth-rates, reaction orders, etc.

---

[1] Presented before the National Academy of Sciences, by invitation of Dr. C. M. Child, under title of "Reaction Gradients and Pigmentation Thresholds in the Feather Germ" (abstract in *Science*, 85 [1937]:59).

[2] This investigation was supported in part by funds from a grant by the Rockefeller Foundation to the University of Chicago in aid of biological research.

The reaction gradient originally described by Lillie and Juhn in the collar of the feather germ is a most beautiful confirmation of the role that C. M. Child has long attributed to purely quantitative, physiological differentials in the developmental processes. The feather germ is remarkable not only because qualitative differentiations evident as different pigmentation products are shown to be localized with respect to quantitative differentials but equally because of the fact that these qualitatively different products are represented in all cells by definite, relatively simple genetic compositions. Here, certainly, is a developmental system for which we may hope eventually to formulate a coherent and rigorous statement of relationship between the discrete date of genetics and the quantitative "organizing" differentials of Child's gradient theory. It is a pleasure to contribute to this number honoring Professor Child a paper which, in common with so much in developmental investigation and theory, must take for granted the pervasive fertility of his conceptions.

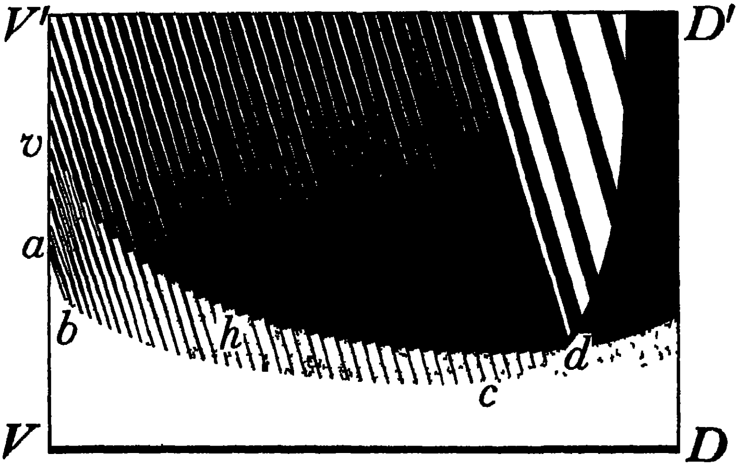


FIG 1.—Diagrammatic representation of one collar limb from a regenerating feather germ. The germ has been split along the ventral superficial axis, VV', and the center of the shaft, *DD'*, the differentiated apical levels of barbs and shafts above *V'D'* have been removed, and also the undifferentiated base of the germ below *VD* Barb primordia originate along *ab*, their bases differentiate along the locus *bc* and are completed at *d* with union of barb-shaft structures Spacing of barbs with growth of shaft is indicated above *d* Locus of simultaneous pigmentation of barb complement is represented by the locus *vd*. Differentiation and pigmentation of shaft are indicated schematically for completion of the diagram.

I. DEVELOPMENTAL RELATIONS IN THE FEATHER GERM

The several orders of induced pigmentation patterns found in regenerated feathers and described below occur in correspondence with definite relations in the developing germ. A brief presentation of essential germinal relations is accordingly called for in this place; detailed accounts have been given by Lillie and Juhn (1932), Greite (1934), Hosker (1936), and Fraps and Juhn (1936).

Figure 1 represents a section cut from the cylindrical base of a germ after development is well under way. The cupped, undifferentiated base of the germ has been removed by cutting along *VD;* the apical, completely differentiated portions of barbs and shaft are cut away along *V'D';* and from the annular band thus formed, one-half of the germ producing a single vane-half of the feather is obtained by splitting along the ventral superficial axis (*VV'*) and through the center of the shaft (*DD'*). The developing region of a complete and wholly symmetrical germ would be represented diagrammatically by producing a mirror image of Figure 1 to the left of *VV'* or to the right of *DD'*.

In the barb complement simultaneously represented in the feather germ (from *b* to *d*, Fig. 1), the youngest primordium is at the ventral face of the collar (at *ab*), the oldest is adjacent to the shaft (at *d*). The apex of the individual barb forms first (*ab*), and successive apicobasal levels arise as the primordium formed at *ab* occupies successive positions along the locus, *bc*. At *d* the base of the completed barb has united with the shaft primordium (union occurring between *c* and *d*), and it is carried from the collar by axially directed growth of the shaft. Growth of the shaft, particularly during the second phase of growth (ballooning of Lillie and Juhn) gives to the barbs their definitive spacing on the shaft. This is indicated—altogether schematically—in Figure 1 by the increasing separation of barbs above *d*.

The region of growth by cell division in the germ includes the undifferentiated base (white of Fig. 1) and a limited extent within barb primordia—up to the distal level of cell division, according to Lillie and Juhn. Axially directed growth causes a continual apicalward prolongation of the undifferentiated zone; the simultaneous projection of barb bases into this zone maintains the general relation shown in Figure 1. It follows in consequence of this axially directed growth that the line of cleavage between the undifferentiated basal tissue and the last barb primordia (*ab*) formed from it will be removed from the base of the germ until a succeeding primordium can form in the same relative position occupied by *ab*, Figure 1.

Since the lines of cleavage between barbs form an angle with the vertical axis, *VV'*, barb bases must move, with continued axial growth of the germ and therefore in length of each barb, from *b* to *c*, where processes of union with the shaft begin (Hosker, 1934). In many germs, however, the angle of cleavage tends to approach the vertical as we follow the locus *bc*. This is the same as saying that the angle of cleavage (*Vab* of Fig. 1) decreases with the level of barb bases above a given base line (*VD* of Fig. 1). The general relationship suggests a primary gradient differential from ventral to dorsal limits of the collar, a differential involving possibly growth vectors (Fraps and Juhn, 1936), as well as rates of growth.

The locus of simultaneous pigmentation in all reactive barb primordia is indicated in Figure 1 by the curve, *vd*. Barb apexes are not pigmented immediately upon their formation, but only after they have reached the relatively high level, *v*, above the base of the germ. There are consequently a number of barb primordia at the ventral limit of the germ that are entirely unpigmented.

It follows from the serial order of origin of barb primordia and their ventrodorsal motion around the collar that the simultaneous pigmentation of all barbs cutting the locus *vd* corresponds to pigmentation of the individual barb at successive apicobasal levels (Lillie and Juhn, 1932). The locus *vd* in the germ appears in the regenerated feather as a line approximately at right angles to the shaft, as *vd* of Figure 2*A–E*: in these representations of single vane-halves, the margin (barb apexes) corresponds to the ventral position in the germ, and barb bases correspond to dorsal position With these simple relations in mind, any pigmentation pattern in the regenerated feather can be referred directly to the developing germ.

2. EXPERIMENTALLY INDUCED ORDERS OF PIGMENTATION

By "order of pigmentation" we refer to the direction—dorsoventral or ventrodorsal—in which an imposed pigmentation pattern displaces an alternative pigmentation formation with increasing hormone concentration. Three orders of pigmentation have been experimentally produced in feathers of the Brown Leghorn male or capon.[3]

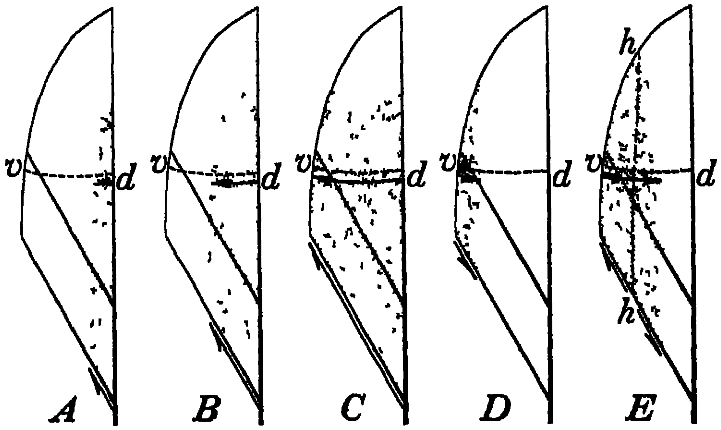


FIG 2—Orders of pigmentation in regenerated feathers Each figure represents a section from a single vane-half with barbs ranged parallel to those shown. The locus of simultaneous pigmentation in the germ (*vd*, Fig 1) is indicated in each section by the transverse broken lines, *vd*. The arrows beneath *vd* give the direction of imposed pigmentation (stippled regions) with increasing hormone concentrations The arrows at the base of the sections give order of pigmentation referred to the individual barb corresponding to levels cut by *vd* *A*, *B*, and *C* dorsoventral order of pigmentation, typical in collars of breast and saddle feathers in reaction to female hormone and thyroxin, respectively. *D* ventrodorsal order of pigmentation with increasing hormone concentration (medial vane-half of secondaries in reaction to female hormone). *E*: complex order of pigmentation, proceeding from a boundary, *hh*, ventrally and dorsally (lateral vane-halves of secondaries)

a) *Dorsoventral pigmentation.*—The order of pigmentation of breast feathers to female hormone and of saddle feathers to thyroxin has been described in detail by Lillie and Juhn (1932). The breast feathers may be taken here as illustrative of this order of reaction. In the male or capon these feathers are solidly black; the subcutaneous injection of female hormone causes the appearance of the brilliantly contrasting "female salmon."

---

[3] Of the three pigmentation orders taken up here, only one, the dorsoventral order, has been described in the literature The ventrodorsal and complex orders of pigmentation were obtained by Dr. Mary Juhn in connection with experiments designed to establish the relation between rate of feather growth and hormone concentration in rapidly growing feathers These results will be published elsewhere. The author wishes to thank Dr. Juhn for placing at his disposal, prior to publication, the extensive material constituting the experimental basis of this paper.

The minimal concentration of female hormone required for the appearance of female salmon in the barbs (leaving out of account the shaft) produces a pattern limited to barb bases. If this relatively low concentration is maintained constant, a longitudinal pattern similar to that indicated in Figure 2*A* is produced (female salmon represented by stipple). This corresponds to reaction at and near the dorsal limit of the germ (to the left of *d*, Fig. 1). A somewhat higher concentration of female hormone maintained at a constant level would produce a larger central area of salmon (Fig. 2*B*), corresponding to reaction in the germ from *d* to *h*. Further increases in the concentration of hormone extend the reaction for salmon pigmentation from *h* toward *v* (Fig. 1) until finally black is displaced entirely from the locus of pigmentation. The resulting feather is completely salmon below the level at which the requisite hormone concentration has been attained (Fig. 2*C*).

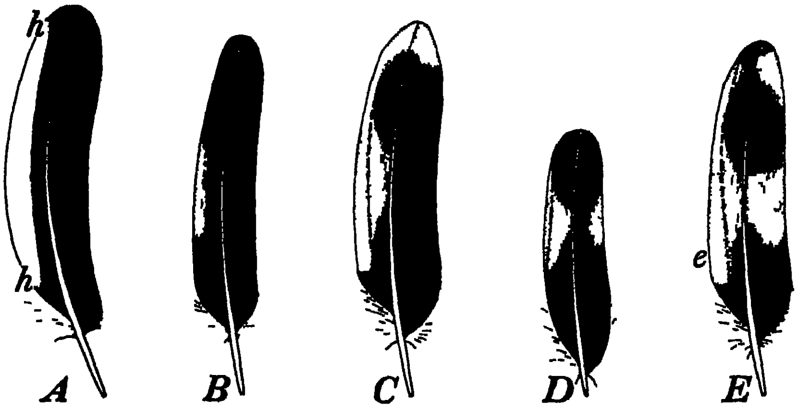


FIG 3.—Normal and modified feathers from the sequence of secondaries, male or capon Brown Leghorn. *A:* normal feather, black in the figure representing black in the feather, white area corresponding to brown in the feather; *hh*, boundary corresponding in mid-vane regions to *h* in Figure 1 *A*, *B*, *C*, and *D*· increasing degrees of reaction to female hormone (stippled areas of figures) in different feathers of the secondary series The longitudinal broken lines in Figures *C*, *D*, and *E* correspond approximately to the normal boundary between brown and black. Details in text.

In the breast feather, then, the order of reaction to increasing concentration of female hormone is strictly dorsoventral. The same order of reaction is typically observed in formation of black under the action of thyroxin in feather germs of the back, saddle, and neck hackle regions of the Brown Leghorn capon (details in Lillie and Juhn, 1932).

b) *Ventrodorsal pigmentation.*—The secondaries of the Brown Leghorn male or capon are typically black with a marginal brown stripe in the lateral vane-half of each feather (Fig. 3*A*, black as represented, brown indicated by white in the lateral [left] vane-half). Female hormone displaces both brown and black, inducing formation of the stippling pattern characteristic of the normal female. Pigmentation of the individual stippled barb occurs as an alternate banding of black and buff. The proportion of black is greater toward the base of the barb, the proportion of buff toward the apex.

Taking into account only the main vane region of the secondaries (excluding, that is, apical formations), increasing concentrations of female hormone effect the formation of stippling in ventrodorsal order *in all normally black regions*. In the medial vane-half (completely black), pigmentation is extended from the ventral limit of the germ to the dorsal limit with increasing hormone concentration (Figs. 2*D* and 3*D*). In the lateral vane-half the extension of stipple with rising hormone concentration proceeds from the boundary between brown and black (*h* of Fig. 1; *hh* of Figs. 2*E* and 3*A*) toward the dorsal limit of the germ, corresponding, of course, with extension toward the shaft in the regenerated feathers (Fig. 3*B*, *C*, *D*, and *E:* observe also that the order of displacement of stipple by black with falling hormone concentration in Fig. 3*C*, *D*, and *E* is from shaft to brown-black boundary).

The most direct evidence that we are dealing with a true ventrodorsal order of reaction in all normally black regions of these feathers lies in the fact that we never find (in the main vane regions) stippled areas adjacent to the shaft with black areas surrounding them. In a few instances the extreme apical levels of barbs in the medial (solidly black) vane-halves remain black even with considerable extension of stipple toward the shaft. But excepting these very limited barb regions, the ventrodorsal relationship appears to be consistently maintained in the displacement of black.

c) Complex pigmentation orders.—In terms of relations within the collar of the developing feather germ, the dorsoventral order of pigmentation (breast feathers) and the ventrodorsal (medial, solidly black vane-half of secondaries) both represent continuous reaction gradients. In the lateral brown-black) vane-half of the secondaries, the reaction for stippling is initiated with minimal hormone concentrations at the boundary between brown and black (*h* of Fig. 1; *hh* of Fig. 3*A*); and with increasing concentrations, stippling is extended from this point both ventrally and dorsally (Fig. 2*E*). This is well brought out in the "mirror-imaging" of the induced stippling on opposite sides of the broken line drawn into Figure 3*C*. The only qualification to be made here is that in a considerable number of feathers the entire brown region appears to have become stippled practically simultaneously, with very little encroachment into the region of black pigmentation. In others, however, marginal areas remain unstippled (brown), as at *e*, Figure 3*E*—clear evidence that the ventral region of the germ has maximal threshold values so far as the displacement of brown is concerned.

The general relation between the concentration of hormone required to effect modification in the several collar limbs, and the direction of modification in each collar limb, can be given in a single graph, Figure 4. Abscissas here represent equivalent collar positions, *dv;* ordinates indicate concentrations. Curve *I* represents the relation between hormone concentration and direction of imposed pigmentation (*dv*) in the dorsoventral order. Curve *II* is the same relation for ventrodorsal order of pigmentation, just the reverse of curve *I*. Curve *III* combines portions of these two curves, with *h* the point of modified pigmentation at minimal hormone concentration.

3. DIFFERENTIAL FUNCTIONS OF PRIMARY GRADIENTS

Lillie and Juhn (1932) interpreted their experimental results, all of which represented dorsoventral reaction in the collar, in terms of a gradient of growth-rates in the regenerating germ. This interpretation was based upon the discovery by Juhn and Gustavson (1930) that the threshold of reaction in feathers of different tracts is proportional to their rates of growth. Juhn, Faulkner, and Gustavson (1931) later showed that the reaction thresholds of feathers within certain contour tracts are likewise proportional to rates of growth during regeneration. In view of these findings, Lillie and Juhn concluded that "it is logical, and indeed necessary, to hold that this principle applies to the parts of a feather as well as to different feathers." Fraps and Juhn (1936) questioned certain data presented by Lillie and Juhn on the growth of barbs; their results do not, however, disprove the existence of small but significant axial rate differentials, nor do they take into account growth differentials in other than the axial dimension.

Assuming that the concentration of hormone required to effect a given reaction is always proportional to rate of growth in the barb primordia, the three orders of pigmentation described above would require the postulation of three fundamentally different growth gradients in different collar limbs. Suppose, on the basis of this assumed proportionality, that we read "growth-rates" instead of "concentrations" in Figure 4. The collar of the breast germ would show gradually increasing growth-rates in dorsoventral order (curve *I*), as was postulated by Lillie and Juhn. The medial (or solidly black) vane-half of secondaries would require the assumption of increasing growth-rates in ventrodorsal order (curve *II*), and the lateral vane-half of the same feathers calls for a curve of growth-rates (curve *III*) with minimal values at an intermediate position, *h*, and increasing rates toward ventral and dorsal limits of the collar.

It seems necessary to suppose that the primary differentials associated with the organization, growth, and development of feather germs are fundamentally alike in all cases, however much germs may differ in scale of organization or in details. The "organizer" properties of the ventral triangle (Lillie and Juhn, 1937) and its field characteristics in certain feathers (Fraps, 1938), the invariable seriation of barb primordia, the increasing physiological age of primordia with ventrodorsal progression through the collar, and the general similarity in conformation of reactive centers for barb bases, for barbulation, and for pigmentation in different germs—all serve to enforce this view. But it is equally certain that the scale of organization of different germs (as breast and secondaries) together with rates of growth and differentiation vary over a relatively great range.

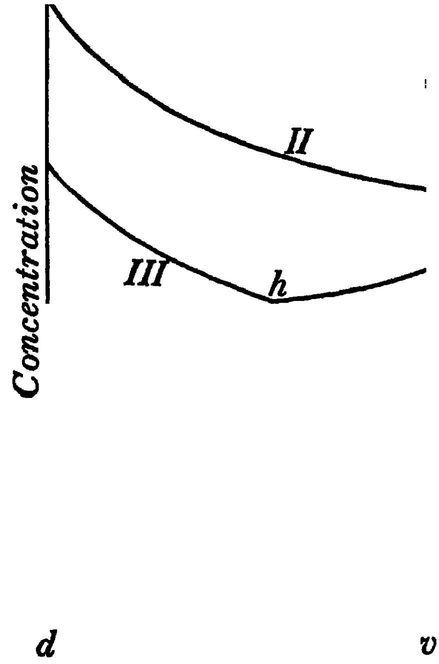


FIG. 4.—Relation between pigmentation loci in the collar (abscissas) and order of reaction to increasing hormone concentrations (ordinates) in different collar limbs. Note that abscissas are ranged in *dorsoventral order*. Curve *I*: breast or saddle germ collar. Curve *II*: the collar from medial vane-half of a secondary, showing the reverse relation of curve *I*. Curve *III*: collar from lateral vane-half of a secondary, combining the relations of curve *I* (*h* to ventral collar limit) and curve *II* (*h* to dorsal collar limit).

Given a system of primary, determinative gradient relations in the collar of feather germs generally (e.g., growth gradients), it follows that secondary processes dependent upon these primary differentials must also be subject to gradient differentials. We may safely assume that these secondary graded processes, or gradient functions, change by differing degrees with change in absolute intensity levels of the primary gradient processes. Two such derivative or secondary gradients will vary also, then, with respect to each other at different absolute levels in the primary gradient system. Wherever two processes (rates, reaction potentials, etc.) are subject to graded intensities in the same or different collar limbs, but differ *with respect to each other* with differing values in an underlying gradient process, they may be denoted differential gradient functions. If, finally, we suppose that certain critical or limiting reactions determining alternative pigmentation processes are themselves differential gradient functions as thus defined, the several pigmentation orders described above can be accounted for satisfactorily without postulating the "reversal" of primary developmental gradients (such as the distribution of growth-rates). Application of this concept to the observed data follows.

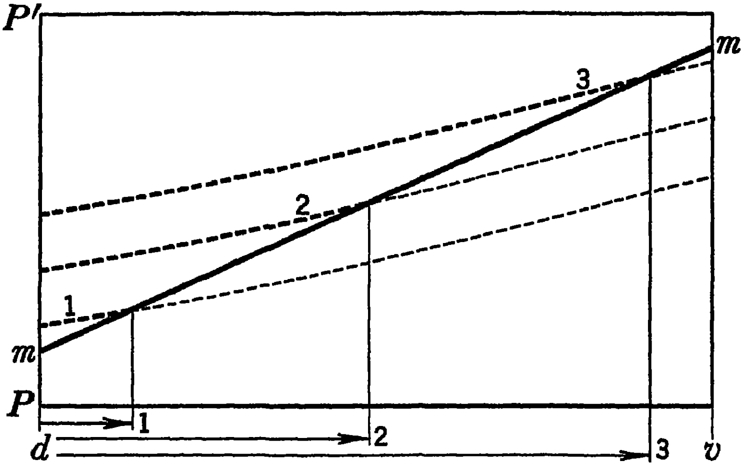


FIG. 5.—Differential gradient functions in the dorsoventral order of imposed pigmentation. Abscissas represent uniformly increasing primary gradient values in the dorsoventral order (from *d* to *v*). Ordinates represent limiting reaction potentials, increasing from *P* to *P'*. Curve *mm* indicates reaction potentials for melanization (in the breast feather). Curve 1: reaction potentials for imposed pigmentation at low concentration of female hormone, displacing melanization reactions from *d* to level 1 on abscissas. Curves 2 and 3: gradients of reaction potentials for imposed pigmentation at two higher concentrations of female hormone, extending the imposed processes to collar levels 2 and 3, respectively.

INTERPRETATION OF THE PIGMENTATION ORDERS

a) *The dorsoventral order.*—The assumption of a direct proportionality between hormone threshold and rate of cell division made by Lillie and Juhn accounted adequately for the dorsoventral extension of imposed pigmentation with rising hormone concentrations in breast and saddle feathers. This interpretation, however, tells us nothing about relations obtaining between the primary gradient (growth-rates) and the conditions determining the partially or completely displaced pigmentation—black in breast feathers, buff in saddle feathers. We accordingly consider this case first.

The differential gradient relations required by the dorsoventral order of pigmentation (breast feathers as example) are given in Figure 5. Let the axis of abscissas, *dv*, represent primary gradient values (as growth-rates), increasing uniformly from *d* to *v*. Let ordinates, *PP′*, represent potentials (intensity, rate, or other values) for the critical, limiting reaction entering into alternative pigmentation processes. If this critical reaction potential for black (in the breast feather) is taken as a direct function of the primary gradient, it may be represented in Figure 5 by the solid line, *mm*. The indicated proportionality between the primary gradient values (abscissas) and the limiting reaction potential for melanization does not necessarily mean that rate of pigmentation, or quantity of pigmentation deposited, increases from dorsal to ventral limits of the primary gradient. This may or may not be true. In the present connection, however, the curve, *mm*, means simply that the reaction potential of the limiting process involved in melanization increases with increasing values of the primary gradient.

When female hormone effects, whether directly or indirectly, an alternative pigmentation formation (salmon instead of black in the breast feather), it is necessary to assume again some proportionality between primary gradient values and potentials of the limiting or critical reaction in the imposed pigmentation processes. This proportionality cannot be identical with that for melanization in the breast feather, since this would mean the simultaneous displacement of black from the entire collar limb at minimal concentrations of hormone inducing any formation of salmon. Since the imposed pigmentation (salmon) appears at minimal concentrations of hormone in dorsally limited regions of the collar, the curve of limiting reaction potentials must have a lesser slope than does the corresponding curve for melanization; only such a proportionality with the primary gradient differentials can have higher potentials in dorsalmost collar regions with minimal hormone concentrations. The required gradient function is represented by curve 1 of Figure 5. Reaction potentials for imposed (salmon) pigmentation processes are higher than are potentials for melanization from *d* to collar level 1, while from level 1 to *v* the relation between the two gradients is reversed. With increased hormone concentration all reaction potentials for salmon are increased (not necessarily uniformly) and we obtain curve 2: all collar loci between *d* and level 2 now react for salmon pigmentation, all loci from collar level 2 to *v* for melanization. Curve 3 represents the gradient of reaction potentials with a concentration of hormone just below the concentration required for complete displacement of melanization processes from the entire collar limb, only ventralmost regions (collar level 3 to *v*, Fig. 5) now having the higher reaction potentials requisite for maintenance of melanization.

Actually, of course, we do not know the absolute reaction potentials for the gradient function represented either by *mm* or by the three imposed curves associated with differing hormone concentrations. We do know that alternative reactions occur dorsally and ventrally from precise loci in the primary gradient (collar levels 1, 2, and 3) at which limiting reaction potentials are equivalent in the secondary gradient functions, and we know also that increasing hormone concentrations must extend the imposed pigmentation processes in dorsoventral order. These relations are satisfied by differential gradient functions of the *order* given in Figure 5.

We have tacitly assumed in the foregoing treatment that the primary gradient values (abscissas of Fig. 5) and the secondary gradient function, *mm*, remain unchanged with differing hormone concentrations. This is probably not the case in breast feathers, and almost certainly not so in the saddle germ under the action of thyroxin. Nevertheless, as long as the general relationship between potentials for alternative pigmentation reaction is maintained in the form represented in Figure 5, the experimentally determined conditions for dorsoventral pigmentation are satisfied.

b) *The ventrodorsal order.*—The ventrodorsal order of pigmentation proceeds from ventral to dorsal limits of the collar with increasing hormone concentration, just the reverse of pigmentation in the breast germ. We consider here only the medial (solidly black) vane-half of the secondaries.

The required relation between secondary gradients representing reaction potentials is given in Figure 6. Abscissas indicate uniformly increasing values in the primary gradient from *d* to *v*; this is the same order of primary gradient differentials followed in Figure 5, but absolute values are presumably much higher in the present case in view of the relatively high growth-rates of regenerating secondaries. Reaction potentials are read as ordinates. Again we assume a proportionality between the secondary gradient function representing limiting reaction potentials for melanization and primary gradient values, giving us the curve *mm* of Figure 6. A relatively low concentration of female hormone induces reaction in ventral collar limits, and we accordingly draw curve 1 *more steeply* than the curve *mm*. This simply means that the limiting or critical potentials for the hormone-induced reaction (stippling) increases relatively more rapidly than do the potentials for melanization with increasing values (reading from *d* to *v*) in the primary gradient. Given this particular differential proportionality between the secondary gradients, the induced pigmentation must appear at the ventral limit of the germ with minimal hormone concentrations, and it will be extended through the collar in ventrodorsal order with increasing concentrations. Thus, in Figure 6 a relatively low hormone concentration establishes the reaction potentials of curve 1, with stippling over the range *v* to collar level 1. An intermediate concentration gives us curve 2, with stippling from *v*

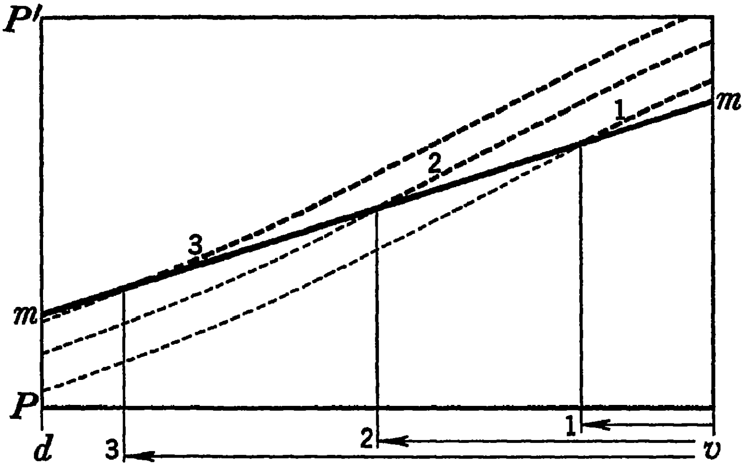


FIG. 6.—Differential gradient functions in the ventrodorsal order of pigmentation. Abscissas: uniformly increasing primary gradient values in dorsoventral order (from *d* to *v*), *the same order* represented in Figure 5, but absolute values understood to be higher. Ordinates: limiting reaction potentials, increasing from *P* to *P'*. Curve *mm*: reaction potentials for melanization. Curve 1: reaction potentials for imposed pigmentation at relatively low concentration of female hormone melanization is displaced from *v* to collar level 1 (reverse of dorsoventral order, Fig. 5) Curves 2 and 3: gradients of reaction potentials for imposed pigmentation at two higher concentrations of female hormone, displacing melanization processes from ventral collar limit to levels 2 and 3, respectively.

to collar level 2; and a relatively high concentration further raises the gradient of reaction potentials for induced pigmentation to the level of curve 3, with stippling imposed over the range *v* to collar level 3. Each of the three curves represents, of course, the stabilized gradient of reaction potentials established by a given concentration of hormone; with gradually increasing concentrations a continuous series of such gradients are imposed in the collar of the germ.

A comparison of Figures 5 and 6 makes it clear that, in terms of differential gradient functions, dorsoventral (Fig. 5) or ventrodorsal (Fig. 6) pigmentation orders represent only differing proportionalities, at differing absolute values in the primary gradient, in the secondary gradients of reaction potentials determining alternative pigmentation processes. This is best seen by comparing the relative slope of curve 1 in each figure with the curve for melanization, *mm*, which it intersects: the relative slopes of these intersecting curves represent the relative proportionalities between alternative reaction potentials over differing ranges of absolute values in the primary gradients (abscissas of Figs. 5 and 6).

c) The complex order.—"Complex" pigmentation orders are incident at a median point in the collar (*h* of Fig. 1) with minimal hormone concentration and proceed toward ventral and dorsal collar limits with increasing concentrations. The certain examples occur in the brown-black vane-halves of the secondaries, with incidence of the imposed pigmentation at the boundary between brown and black reactions. We must, accordingly, begin with some relationship beween reaction potentials for the alternative pigmentation processes of the normal feather. The most likely interpretation is that reaction potentials for these alternatives represent differential gradient functions in exactly the sense that experimentally imposed and "normal" reaction potentials have already been related.

In Figure 7 abscissas indicate increasing values in the primary gradient from dorsal to ventral collar limits; ordinates represent reaction potentials. Let the curve *mm* represent the gradient of reaction potentials for melanization, and *rr* the gradient of reaction potentials for brown pigmentation. Melanization will then occur from the dorsal limit of the collar, *d*, to the intermediate position, *h;* and from *h* to the ventral collar limit, brown pigmentation processes are completed (cf. Fig. 1). The fact that the boundary between brown and black approaches the shaft in the order of increasing growth-rates through the sequence of secondaries suggests certainly that some such purely quantitative relation determines alternative reactions.

If now we suppose the gradient of reaction potentials for the hormone-induced pigmentation processes (stippling) to be steeper than that for melanization, but less steep than that for formation of brown, the initial point of imposed pigmentation must occur at the boundary between brown and black reactions. This condition is satisfied in Figure 7 by any straight line taking its origin between *m* and *r* and passing through *o*. The experimentally determined patterns suggest, however, that the concentration of hormone required for modification of the brown-producing collar regions is much less than that required for equivalent modification in the black-producing regions. We accordingly draw *ss* through *o* to make the angle *sor* relatively small and the angle *som* relatively great. In other words, we represent the gradient of reaction potentials for induced pigmentation as differing relatively little from the gradient of reaction potentials for brown pigmentation. If the two gradients of reaction potentials (for induced and for brown pigmentation) represented identical functions of the primary gradient, brown should be displaced simultaneously from collar level *h* to the ventral limit of the collar with no appreciable displacement from *h* toward the dorsal limit (*rr* and *ss* of Fig. 7 would, in this instance, be identical).

A second gradient of reaction potentials, established by a higher concentration of hormone, is indicated by curve 2 of Figure 7. Induced pigmentation processes have completely displaced brown; and the order of displacement is from collar level *h* toward *v*, the ventral limit of the collar (dorso-ventral order). Melanization processes are displaced, however, over only the limited range *h* to collar level 2, and in ventrodorsal order. With a considerably higher concentration of hormone we obtain curve 3, leaving only a dorsally limited collar region (level 3 to *d*, Fig. 7) in which melanization continues. Still higher concentrations of course displace melanization from this region also, producing reaction for stippling through the entire extent of the collar.

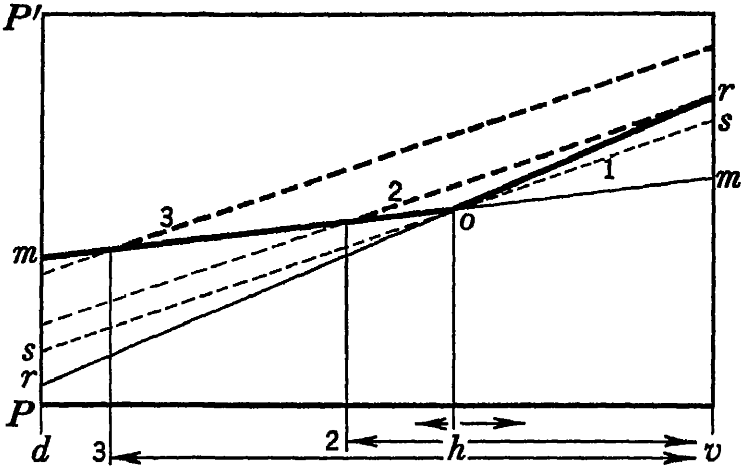


FIG. 7.—Differential gradient functions in the "complex" order of pigmentation. Abscissas: primary gradient values in increasing dorsoventral order (from *d* to *v*). Ordinates: limiting reaction potentials, increasing from *P* to *P'*. Curve *mm:* reaction potentials for melanization in the normal feather. Curve *rr:* reaction potentials for brown in the normal feather. These two gradients of the normal reaction potentials, intersecting at *o*, refer the boundary of normal brown-black pigmentation to the collar level *h*, corresponding to *h* of Figure 1. Curve *ss:* gradient of reaction potentials for minimal concentration of hormone effecting imposed reaction (stipple). Curves 2 and 3: gradients of reaction potentials for higher concentrations of hormone. Curve 2 completely displaces processes for brown pigmentation in dorsoventral order (from *h* to *v*), and displaces melanization in ventrodorsal order (from *h* to level 2). Curve 3 further displaces melanization in ventrodorsal order to collar level 3.

The plumage of the Brown Leghorn male or capon exhibits a wide range of pigmentation combinations, only one of which—the brown-black combination in the secondaries—has just been considered in terms of differential gradient functions. A simple and wholly theoretical application of differential functions over a relatively great range of values in an assumed primary gradient will at least suggest a basis for the observed diversity of pigmentation combinations generally.

Abscissas of Figure 8 indicate increasing values (from *G* to *G'*) in an assumed primary gradient; all values found in an array of collar limbs are included on this single axis of abscissas. Reaction potentials are read as ordinates. Let the curve mm represent the gradient of reaction potentials for melanization, and curve rr the gradient of reaction potentials for an alternative pigmentation—say red in order to distinguish this theoretical case from the Brown Leghorn pattern considered above. We assume that the general physiological conditions of the bird remain uniform, which is to say that the two gradients for alternative reaction potentials are stable for the case under discussion.

In terms of Figure 8, then, all collar limbs with primary gradient values falling between G and h', and h and h'', must produce red pigmentation; all collar limbs with primary gradient values falling between h' and h, or h'' and G', can react only for melanization. Any collar limb including the values h', h, or h'', corresponding to equivalent reaction potentials o', o, or o'', will produce complex pigmentation patterns. Collar limbs $d'v'$ and $d''v''$ must each react for red pigmentation from a median collar level, h' or h'', to the dorsal limits of the collars, d' and d''. From the same medial point, h' or h'', to the ventral collar limits, v' and v'', melinization takes place. Thus, at two extremes of primary gradient values we should obtain feathers with an identical complex pattern, a red central region with black marginal areas. The collar limits dv (Fig. 7) will also produce a complex pigmentation pattern, but in this case the feather will have a black central region with red forming marginally, as brown does in the lateral vane-half of secondaries in the Brown Leghorn (Fig. 3*A*).

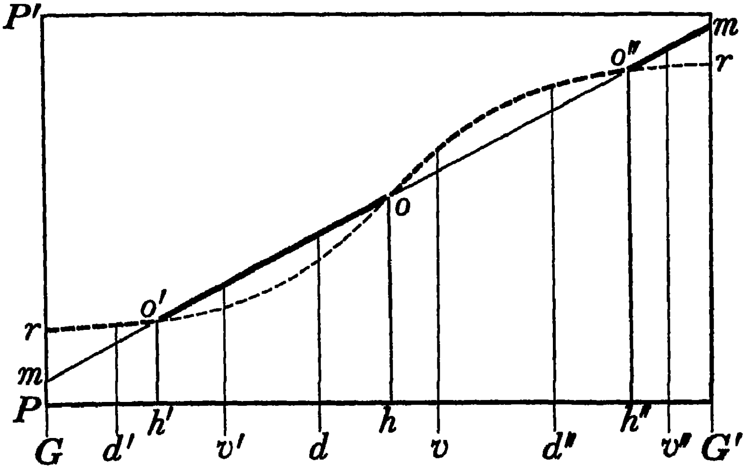


FIG. 8.—Theoretical relation between continuous differential gradient functions required for "reverse" and complex pigmentation orders in different feathers. Abscissas: increasing values in primary differentials (from G to G') for an array of collar limbs. Ordinates: limiting reaction potentials. Curve *mm:* reaction potentials for melanization. Curve *rr:* reaction potentials for red pigmentation. All collar limbs with primary gradient values falling between G and h', and h and h'', can produce only red pigmentation; all collar limbs with primary gradient values falling between h' and h, and h'' and G, can react only for melanization. Collar limbs including h', h, or h'', corresponding to equivalent reaction potentials o', o and o'', will exhibit complex pigmentation orders. Collar limbs $d'v'$, and $d''v''$: red dorsally, black ventrally. Collar limb dv: black dorsally, red ventrally.

4. DISCUSSION

The obvious limitation in the foregoing interpretations of the several experimentally induced pigmentation orders is that we cannot define with precision the limiting reaction potentials treated as differential gradient functions. From this eventual point of view the representations given in this paper are formal, as they must remain to varying degree until our knowledge of physical and chemical processes constituting the chain of events from initial genic action to final deposition of pigment has been greatly extended. In the meantime there is at hand the considerable body of physiologically significant relationships that are satisfactorily accounted for in principle. The identification and quantitative formulation of the postulated limiting reaction potentials becomes a main problem for future investigation.

From a genetic point of view, however, the postulation of an independent gradient of critical reaction potentials for each pigmentation process is by no means an arbitrary assumption. In sexually dimorphic plumages, at least, the colors that can be produced by hormones are genetically represented in all cells of reactive follicles (Danforth, 1933). The same is presumably true of those pigmentation reactions that are independent of hormone action or below the threshold of hormone effect. Diversity of genetic resultants appearing as pigmentation alternatives must then be due to extra-genic differentials, and in the present paper these have been described as differential functions of primary gradients. This assumes, of course, differing reaction chains for differing pigmentation products. The existence of such differing reaction chains must certainly be granted wherever differing genic action is involved, for each gene or genic complex potentially capable of producing an independent or differentiated resultant must do so through a sequence of reactions differing in some degree from the series of reactions that can be attributed to any other gene or genic complex.

Differential gradient functions represent the relationship between gradients of limiting or critical reactions in two such independently initiated reaction series. It is not necessary to assume that the action of a gene initiating a reaction chain is itself a function of primary gradient differentials; this may or may not be the case. It is only necessary to suppose that critical reactions in the series become subject to the primary gradients before completion of the pigmentation process. This may occur at any time after initial genic action, and must occur whenever the reaction chain involves processes (or substances) which are directly or indirectly functions (as distributions of rates, substances, etc.) of primary gradient differentials. And, remembering the "discrete" genic initiation of alternative reaction series, it seems necessary to hold that component reactions in each chain cannot ordinarily be identical functions of the primary gradients but will, on the contrary, exhibit a differential relationship of the order represented in the several figures previously considered.

The *order* of pigmentation of feathers under varying hormone concentrations has been studied quantitatively in only a single bird, the Brown Leghorn, and in any detail for only certain of the feather tracts. The results obtained with this bird demonstrate beyond all doubt the fundamental role of purely quantitative differentials in the localization of pigmentation reactions in the individual feather. This is true altogether apart from the interpretation placed upon the observed quantitative relationships. The further analysis of these differentials and the testing of hypotheses designed to interpret them call for application of the quantitative procedures of Lillie and Juhn to plumages of differing genetic compositions. A comprehensive investigation of differing genetic compositions by these methods might reasonably be expected to throw some light on the mechanism of genic action as well as to clarify the nature of the differentials through which the localization of resultants is effected.

5. SUMMARY

1. Three orders of pigmentation induced in feathers of the Brown Leghorn male or capon with increasing hormone concentration are described. These are, in terms of loci in the collar of the developing germ: (*a*) the dorsoventral order previously described in detail by Lillie and Juhn; (*b*) the ventrodorsal order, which is completely the reverse of (*a*); and (*c*) a complex order, proceeding from a medial point in the collar toward both ventral and dorsal collar limits.

2. These orders of pigmentation are interpreted in terms of differential gradient functions, that is, in terms of secondary gradients in the limiting reaction potentials for alternative pigmentation processes. Each of these secondary gradients, or gradient functions, is assumed to vary by differing proportionalities with a given change in primary gradient differentials, which are assumed to remain always the same in direction although varying in absolute values in collar limbs of different germs.

LITERATURE CITED

DANFORTH, C. H. 1933. Genetic factors in the response of feather follicles to thyroxin and theelin. Jour. Exper. Zoöl., 65:183–97.

FRAPS, RICHARD M. 1938. Effects of thyroxin and female hormone on one phase of saddle feather development. Proc. Soc. Exper. Biol. and Med., in press.

FRAPS, RICHARD M., and JUHN, MARY. 1936. Developmental analysis in plumage. II. Plumage configurations and the mechanism of feather development. Physiol. Zoöl., 9:319–77.

GREITE, WALTER. 1934. Die Strukturbildung der Vogelfeder und ihre Pigmentierung durch Melanine. Zeitschr. f. wiss. Zool., 145:283–336.

HOSKER, ANNE. 1934. Bilateral gyandromorphism in feathers. Nature, 133:382–83.

———. 1936. Studies on the epidermal structures of birds. Phil Trans. Roy. Soc. London, B, 226:143–88.

JUHN, MARY; FAULKNER, G. H.; and GUSTAVSON, R. G. 1931. The correlation of rates of growth and hormone threshold in the feathers of fowls. Jour. Exper. Zoöl., 58:69–111.

JUHN, MARY, and GUSTAVSON, R. G. 1930. The production of female genital subsidiary characters and plumage sex characters by injection of human placental hormone in fowls. *Ibid.*, 56:31–61.

LILLIE, FRANK R. 1932. The physiology of feather pattern. Wilson Bull., 44:193–211.

LILLIE, FRANK R., and JUHN, MARY. 1932. The physiology of development of feathers. I. Growth-rate and pattern in the individual feather. Physiol. Zoöl., 5:124–84.

———. 1937. The origin of the after-feather in fowl: a process of twinning. Science, 86:38–39.

SINGLE AND COMBINED EFFECTS OF CYANIDE AND METHYLENE BLUE ON RECONSTITUTION IN EUPLANARIA DOROTOCEPHALA[1]

(Eight figures)

OLIN RULON
Univeısity of Chicago and Wayne University

IT HAS long been known that cyanide exerts its chief effects on living protoplasm by inhibiting respiratory metabolism. It is also known that cyanide may retard cell division and growth. Certain experiments by various workers (Hug, 1932; Brooks, 1932, 1933; Bodine and Boell, 1936, 1937; and others) have shown that methylene blue, in certain concentrations, will antagonize the poisonous effects of cyanide and restore the normal respiration in whole adult animals and in developing embryos. In contrast with the effects of cyanide, methylene blue has been found, in certain cases, to increase the rate of respiratory metabolism above the normal.

Child (1916) found that it was possible to alter and control the character of the head formed and the frequency of head formation in pieces of *Euplanaria* through the use of low concentrations of KCN. It was apparent from his experiments that cyanide not only inhibited the growth of new tissue but exerted inhibitory effects on other factors related to the reconstitutional process. With this work in mind the present research was undertaken in an attempt to determine additional effects of cyanide, the effects of methylene blue, and the combined effects of the two on reconstitutional development.

MATERIAL AND METHODS

The material used in this investigation was the common flatworm of the Chicago region, *Euplanaria dorotocephala*. The animals were usually kept in the laboratory for several weeks after collection and were not fed for approximately 2 weeks before the experiments were begun. Animals, 14–16 and 15–17 mm. in length, were used; and of these, only pieces from the anterior zooid between the mouth and head were tested. The pieces were approximately one-eighth the length of the entire animal, exclusive of the head, and were labeled from anterior to posterior: *A*, *B*, *C*, and *D* (Fig 1). In all experiments lots of 50 pieces from each region were used. These lots were obtained by sectioning 65 animals and selecting 50 pieces of most nearly the same size from each region. The pieces were then placed in the test solutions in 1-liter flasks for varying intervals of time after section. All experiments in testing the effects of methylene blue and cyanide on reconstitution were controlled by the reconstitution of similar pieces in well water.

Animals reconstituting in the test and control solutions were kept in the dark at room temperatures for the entire reconstitutional period. After the reconstitutional period, which lasted approximately 2 weeks, the animals were removed from the flasks and examined with a binocular microscope to determine the type of head and other reconstitutional phenomena. The different head types have been classified by Child (1911*a*, 1911*b*, 1911*c*, 1912, 1914*a*, 1914*b*, 1916, 1920, 1924) on the degree of inhibition,

---

[1] Support for this work was provided from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago.

and this classification is followed in this work. Briefly stated, the different types are: (1) *normal*—with triangular head, auricles at lateral margins, and two symmetrically placed eyes; (2) *teratophthalmic*—with triangular head and auricles in normal position but with eyes showing all degrees of approximation to the median plane from two distinct eyes with pigment connected up to complete cyclopia; (3) *teratomorphic*—with head more or less rounded in outline and with auricles more or less anterior and showing all degrees of approximation to the median plane up to a single median auricle and with a single, or apparently single, median eye; (4) *anophthalmic*—with head rudimentary, without eyes, and with or without a single median auricle; (5) *acephalic* with head completely absent.

Child and Watanabe (1935) have given a fairly reliable method for the evaluation of the approximate average degree of head inhibition of pieces from a particular level and of a given size. In this method each type of head is designated by an arbitrary number that corresponds roughly to the degree of inhibition. The arbitrary values of each type are as follows: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20. The head-frequency index (designated as "mean" by Child and Watanabe) is calculated for any particular lot of worms by multiplying the number of each head type by its arbitrary number and dividing the sum of all by the total number of living animals. It is easily seen that animals reconstituting from *A* levels, which usually develop almost 100 per cent normal heads, will have an index approaching 100, while animals reconstituting from *D* levels, which usually give a high percentage of strongly inhibited head types under control conditions, will have an index approaching 20.

Since many experiments in this laboratory by various workers have shown that a comparison of head-frequency indices of test and control experiments gives a reliable method for discerning the effects of various agents on reconstitution, graphs showing the indices plotted against body-level are used throughout this paper in the presentation of the data.

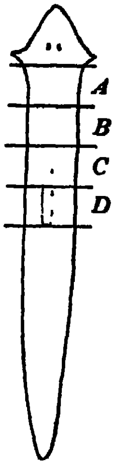


FIG. 1.—Levels *A*, *B*, *C*, and *D* used in all head-frequency experiments. Each section is approximately one-eighth the length of the animal, exclusive of the head. Head and region posterior to the mouth discarded.

EXPERIMENTAL DATA

1. *The effect of KCN on head frequency.*—Ten different experiments were performed on different stocks of worms in an effort to determine additional effects of cyanide on head frequency. Each experiment involved 50 test and 50 control pieces from each of the four different levels. In the tests the pieces were placed in M/100,000 KCN immediately after section, and kept in this solution for 72 hours. After this initial exposure to the cyanide the pieces were transferred to well water for the remainder of the experimental period. (Pieces left in this concentration of KCN for the entire reconstitutional period are so pigmented and inhibited that it is next to impossible to catalogue the different head types.)

In seven of the ten experiments testing the effect of cyanide on head frequency, the controls showed a relatively high head frequency, while the remaining three showed a considerably lower head frequency. The differences in head frequency in different stocks have been dealt with in previous work (Rulon, 1936). Of the seven experiments with a high head frequency in the controls, the tests in every case (in pieces from *B*, *C*, and *D* levels) showed a lower head frequency. This is shown graphically in Figure 2. In the graph the reconstituted forms from the 350 control animals are averaged together to give curve *L*, while the 350 test animals are shown as curve *M*.[3]

In the three experiments in which the controls showed a low head frequency, the tests behaved somewhat differently. Pieces from *A*, *B*, and *C* levels treated with cyanide give a lower head frequency than the controls, while pieces from *D* levels show a distinctly higher head frequency. This is shown graphically in Figure 3, in which the 150 test animals are shown by curve *M* and the control animals by curve *L*.

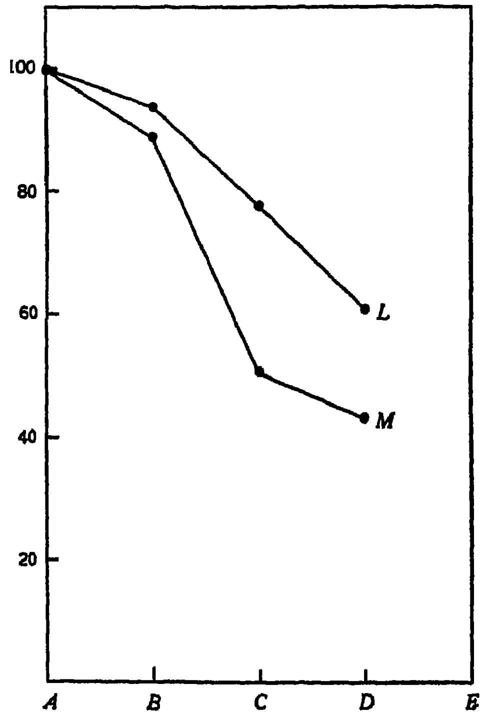


FIG 2

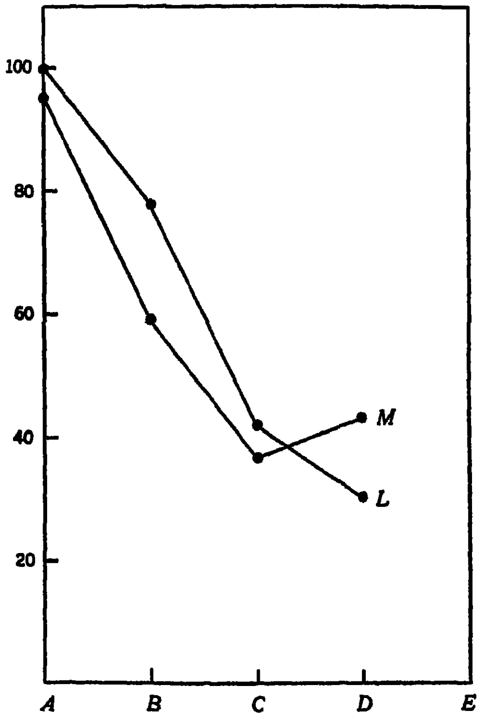


FIG 3

FIG 2.—Combined data of seven experiments showing the effects of cyanide on head frequency of animals having a high head frequency in the controls. *L* = control. *M* = test.

FIG 3.—Combined data of three experiments showing the effects of cyanide on head frequency of animals having a low head frequency in the controls. *L* = control *M* = test.

2. *The effect of methylene blue on head frequency.*—In order to determine a suitable concentration, experiments were run in which intact animals were submitted to various solutions of the dye for different intervals of time. It was found, in general, that intact

---

[3] All graphs are plotted with the head-frequency indices as ordinates and body levels as abscissas.

animals (14–16 mm. in length) could live for 2–3 days in 1/100,000 and for 5–7 days in 1/200,000 solution. These animals became heavily stained, inactive, and strongly insensitive before cytolysis finally set in. Short pieces (one-fourths of the anterior zooid) were more susceptible to these concentrations; and, although they could live and reconstitute in 1/500,000, the best results were obtained by allowing the pieces to stay in 1/200,000–1/300,000 methylene-blue solution for 72 hours and then returning them to well water for the remainder of the reconstitutional period. At the end of 72 hours in the dye the pieces are deeply stained and are relatively insensitive to external stimuli.

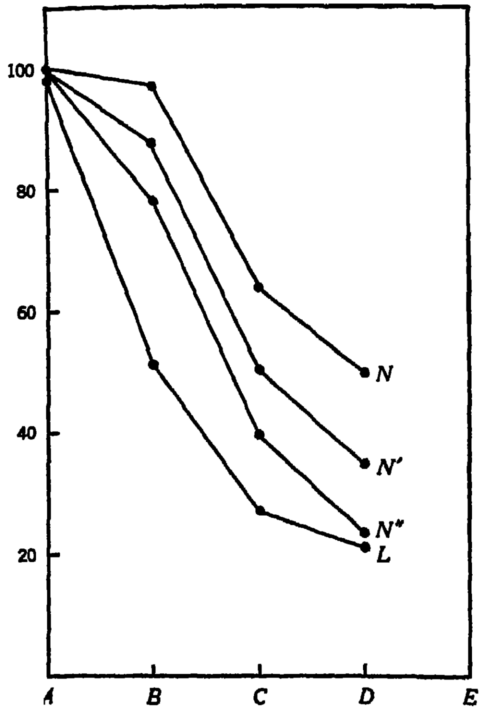


Fig 4

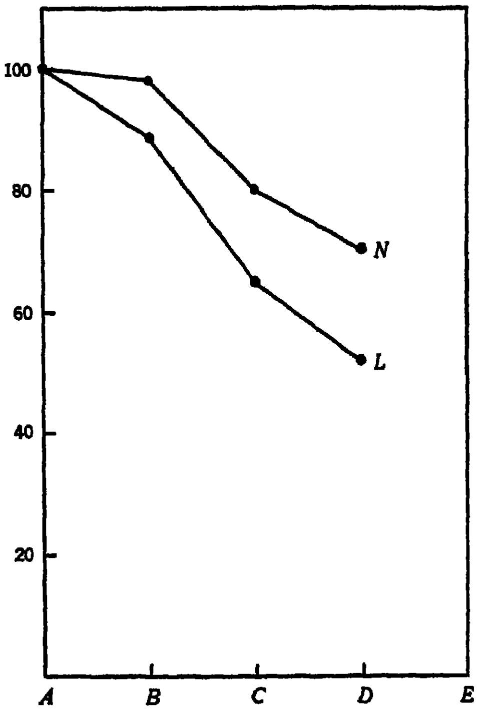


Fig 5

Fig 4.—Data of one experiment showing the effects of different concentrations of methylene blue on head frequency. L = control. N = 1/200,000 methylene blue for 60 hours. N' = 1/500,000 methylene blue for entire reconstitutional period. N'' = 1/1,000,000 methylene blue for entire reconstitutional period.

Fig. 5.—Combined data of nine experiments showing the effects of 72 hours' exposure to 1/200,000–1/300,000 methylene blue on head frequency. L = control. N = test.

They retain considerable of their blue stain after return to water, and even 2 weeks later the gut cells may contain many blue granules.

A comparison of long-time exposure to weak solutions and short-time exposure to stronger solutions of methylene blue is shown graphically in Figure 4. Fifty pieces were used for each level in the control and each test. Curve L represents the head frequency of the control in well water. The head frequency of animals reconstituting for the first 60 hours in 1/200,000 methylene blue and for the remainder of the period in well water is shown by curve *N*. Pieces reconstituting for the entire period in 1/500,000 methylene blue are shown by curve *N′* and for the entire period in 1/1,000,000 by curve *N″*. These experiments show quite definitely that methylene blue increases the head frequency over a range of concentrations but that short-time exposure to higher concentrations is more effective.

Nine experiments were performed in which the tests were submitted to 1/200,000–1/300,000 methylene blue for the first 72 hours after section. In every case pieces from *B*, *C*, and *D* levels treated with methylene blue gave a higher head frequency than the controls for similar levels. In general, pieces from lots with a low head frequency showed a greater increase in head frequency when treated with methylene blue than did pieces from lots that gave a high head frequency in the controls. In *A* pieces no effects of the methylene blue could be detected in head frequency, since pieces from this level in both the tests and controls reconstituted nearly 100 per cent normal heads. The head-

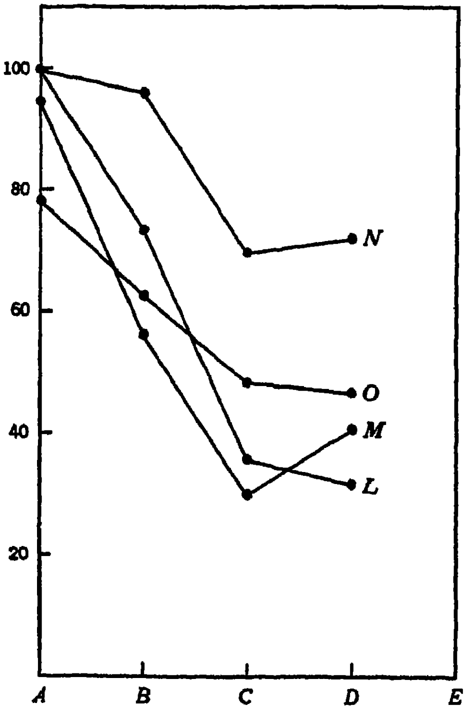


FIG. 6

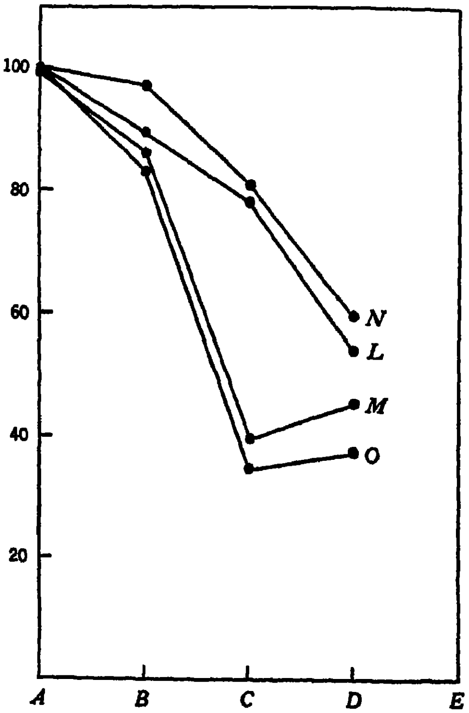


FIG. 7

FIG. 6.—Combined data of two experiments showing the single and combined effects of 1/300,000 methylene blue and M/100,000 KCN on head frequency of animals with a low-control head frequency after 72 hours' exposure. *L*=control. *M*=cyanide. *N*=methylene blue. *O*=combined solution of cyanide and methylene blue.

FIG. 7.—Combined data of two experiments showing the single and combined effects of 1/300,000 methylene blue and M/100,000 KCN on head frequency of animals with a high-control head frequency after 72 hours' exposure. *L*=control. *M*=cyanide. *N*=methylene blue. *O*=combined solution of cyanide and methylene blue.

frequency indices of these nine experiments (450 control and 450 test animals) are averaged and shown graphically in Figure 5. Curve *L* represents the average head frequency of the controls; and curve *N*, the tests.

It has been noted that many of the animals reconstituting after exposure to methylene blue appear somewhat swollen and in many cases considerably more pigmented than in the controls. These phenomena are of more common occurrence in pieces from the more anterior levels.

3. *The combined effect of methylene blue and KCN on head frequency.*—Two series of experiments were performed in which stocks showing a low head frequency in the controls were used (Fig. 6, curve *L*). Such animals gave results similar to those shown in Figure 3 when treated with M/100,000 KCN for 72 hours after section (Fig. 6, curve *M*). It will be noted that the head frequency of animals from *A*, *B*, and *C* levels is below, and of *D* level above, that of the controls. Similar pieces treated with 1/300,000 methylene blue show a tremendous increase in head frequency at *B*, *C*, and *D* levels (Fig. 6, curve *N*). Pieces submitted to the combined solution (M/100,000 KCN; 1/300,000 methylene blue) for 72 hours after section gave very interesting head frequencies (Fig. 6, curve *O*). It is to be noted that *A* pieces show a definitely lowered head frequency when treated

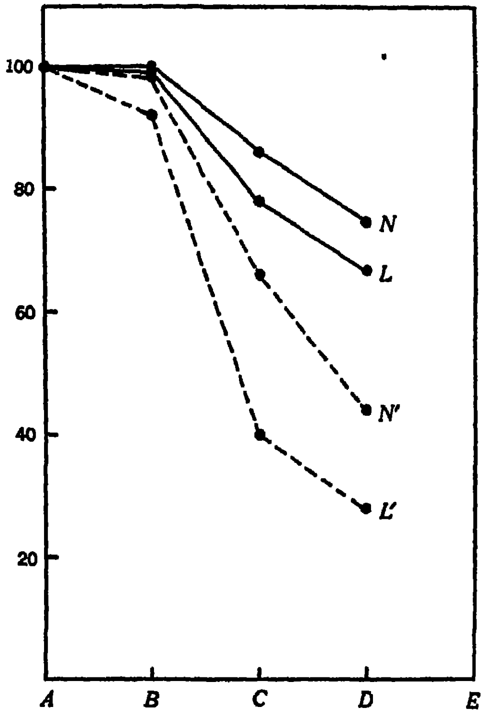


FIG. 8.—Data of one experiment showing the effects on head frequency by 6 days' exposure of intact animals to M/100,000 KCN prior to section, and the recovery in well water and methylene blue after section. Animals treated with cyanide shown by broken lines. *L*=untreated control. *L'*=cyanide-treated animals reconstituted in well water. *N*=untreated methylene blue. *N'*=cyanide-treated animals exposed to methylene blue after section.

with this combination. The head frequency of animals from *B* pieces is higher than that of animals which have been treated with KCN alone but lower than the controls. The head frequencies of *C* and *D* pieces are distinctly higher than the controls or those treated with KCN.

Two series of experiments were performed in which the stocks showed a relatively high-control head frequency (Fig. 7, curve *L*). These animals gave results similar to those shown in Figure 2 when treated for 72 hours with M/100,000 KCN, in that the head frequencies of pieces from *B*, *C*, and *D* levels are below the controls (Fig. 7, curve *M*). Methylene blue, as in all other experiments, increased the head frequency of pieces from *B*, *C*, and *D* levels. The combined solution (M/100,000 KCN; 1/300,000 methylene blue) gave results, however, that were considerably different than those from stocks which show a low head frequency in the controls. Pieces from *A* level were apparently unaffected, while *B*, *C*, and *D* pieces gave head frequencies *below* those of cyanide alone (Fig. 7, curve *O*).

One series of experiments was performed in which a lot of 15–17 mm. worms, showing a very high head frequency in the controls, was placed in M/100,000 KCN for 6 days prior to section. After this initial exposure of intact animals to the cyanide solution, they were sectioned, as in the preceding experiments (anterior zooid cut in fourths). The pieces from the different levels were then allowed to reconstitute (1) in well water and (2) in 1/200,000 methylene blue for 72 hours and then in well water. Equal numbers (50 worms for each test and control) of the same size and from the same stock, but without the cyanide treatment, were allowed to reconstitute in well water and 1/200,000 methylene blue (72 hours). The head frequency data of this experiment are shown graphically in Figure 8. The animals treated with cyanide prior to section are represented by dotted lines, while those untreated with this agent are shown by solid lines. It is readily seen that animals treated with cyanide before section and then allowed to reconstitute in well water (curve *L'*) show a distinctly lower head frequency at *B*, *C*, and *D* levels than untreated animals in well water (curve *L*). Likewise, cyanide-treated animals reconstituting for 72 hours in methylene blue show a lower head frequency (curve *N'*) than untreated animals with the same exposure to methylene blue (curve *N*). The important point to consider in this experiment, however, is the fact that methylene blue increases the head frequency of cyanide-treated worms over the treated worms which have reconstituted in well water. In other words, methylene blue appears to partially antagonize the effects of an initial exposure to cyanide.

DISCUSSION

It has been pointed out in previous work that, under normal conditions, at least two different factors determine whether or not short transverse pieces will reconstitute a head. The first of these factors has to do with the anteroposterior gradient of the intact animal. When a single section is made in *Euplanaria* at any level, a new head will be reconstituted. In other words, if the anterior portion of the animal is removed, a new head will grow on the posterior portion. The rate of reconstitution of the new head is dependent upon the level at which the section is made (Watanabe, 1935*a*). When the section is made at the anterior level of the first zooid, the rate is more rapid than when the section is made at a more posterior level of the same zooid. Such experiments show an anteroposterior gradient in rate of head development.

The second factor affecting head development is of a different nature. When short transverse pieces from the different levels reconstitute, it has been found that pieces of the same size but from different levels will not reconstitute in a like manner. Pieces, one-eighth the length of the intact animal, from a region just posterior to the head, will usually reconstitute almost 100 per cent normal heads; but pieces of the same length from more posterior levels of the same zooid (*D* pieces) usually reconstitute a high percentage of headless forms or forms with inhibited heads. It has been pointed out in previous work that the inhibition of heads in short transverse pieces is due to some factor or factors which arise at the posterior cut surface of the piece. This head-inhibiting factor may last for several days during the early stages of tail formation. Child and Watanabe (1935) and Watanabe (1935*b*) have shown that the head-inhibiting factor is probably conducted from its posterior point of origin to the head-forming region by way of the ventral nerve cords. If the head-inhibiting factor can be blocked or eliminated, or the head-forming cells near the anterior cut surface be given a start in the reconstitutional process (i.e., by delaying posterior section by 12 hours or more), animals with normal heads may be caused to reconstitute from short pieces of any level.

Since the rate of head formation is highest at more anterior levels, it has been suggested that the reason short pieces from anterior levels reconstitute normal heads while pieces of the same size from more posterior levels do not is because the more active head-forming regions of the anterior pieces may get out from under control of the posterior inhibiting factors, whereas the less active head-forming regions of posterior pieces are unable to do so. Of course, since we have no way for complete analysis, it may equally be true that the posterior head-inhibiting factor may be more active at the more posterior levels.

A large number of different agents have been used to increase the head frequency of short posterior pieces of *Euplanaria* by blocking, or in some manner decreasing, the action of the posterior head-inhibiting factor (Child, *1916*; Buchanan, *1922*; Hinrichs, 1924; Rulon, 1936, 1937; Miller, 1937). Evidence has also been presented which indicates that head frequency may be increased through the stimulation of the head-forming cells. Various agents, such as metabolic end-products, ethyl alcohol, dilute cyanide (Child, 1911*a*, 1912, 1916), anesthetics (Buchanan, 1922), and caffeine (Hinrichs, 1924), may decrease the head frequency of pieces from anterior levels and long pieces with certain exposure periods and concentrations. This inhibition is apparently due to a direct inhibitory effect of the agent on the head-forming cells, the different types of inhibited heads indicating that the most active regions are the most retarded in development.

Child and others have also found that, while cyanide and other agents may lower the head frequency of short anterior pieces, it may, at the same time, increase the head frequency of more posterior pieces. This increase is apparently due to partial elimination or blocking of the posterior inhibiting factor, while the head-forming cells, because of their normal lower activity, are less susceptible than the head-forming cells of more anterior regions.

In our experiments it has been found that different stocks, apparently because of a difference in physiological condition, respond somewhat differently to cyanide treatment. Animals which show a relatively high head frequency in the controls (Fig. 2) show a lowering in head frequency in pieces from *B*, *C*, and *D* levels. This decrease is particularly marked in *C* and *D* pieces. On the other hand, *A* pieces are apparently not affected by the treatment. It must be remembered, however, that the pieces were in the agent for only 72 hours after section and that at the end of this time they were returned to well water. (If they had been kept for the entire period in the cyanide solution, very little, if any, new tissue would have developed.) Apparently, after the 72 hours' initial exposure the *A* pieces, presumably because of their initial higher activity, were able to recover, while more posterior pieces were unable to do so. These data then appear to support Child's hypothesis of differential recovery. At the same time, these data show the direct inhibitory effect of cyanide on the head-forming cells of pieces from the posterior levels.

Stocks which show a low head frequency under control conditions behave somewhat differently when pieces from the different levels are exposed to cyanide for 72 hours (Fig. 3). Pieces from *A*, *B*, and *C* levels respond by showing a slight but definite decrease in head frequency. Pieces from level *D*, however, show an increase in head frequency. Apparently, in these stocks *A* pieces, because of a different physiological condition, are unable to recover fully from the inhibitory effects of the agent. In *D* pieces the head frequency is apparently increased by a blocking or partial elimination of the posterior head-inhibiting factor, while the head-forming cells, because of a low activity at this level, are not inhibited as strongly by the direct action of the agent. From a consideration of all data it would then appear that the chief effect of cyanide is the inhibition of growth in the head-forming region, although other factors may enter in which tend to obscure the situation.

In considering the effects of methylene blue on head frequency in short transverse pieces of *Euplanaria*, it was found that in every experiment methylene blue brought about an increase in head frequency in pieces from *B*, *C*, and *D* levels. It was also found that stocks with a low head frequency in the controls were affected to a greater extent by this agent than were stocks with a high head frequency. At first it was thought that methylene blue was acting on planarian tissue in a manner directly opposite to that of cyanide. In other words, cyanide lowered the activity of the head-forming cells while methylene blue increased this activity. This assumption may be true in part, for no experiments have as yet been performed testing the respiratory metabolism of the pieces exposed to these two different agents. From the reconstitutional standpoint, however, it appeared that methylene blue, in the concentrations used, acted more as an inhibitory agent. Pieces treated with 1/200,000–1/300,000 methylene blue did not appear to have been stimulated as regards reconstitution. This was particularly true of pieces from the more anterior levels, where the amount of new tissue and the size of the eyespots appeared to be less than in the controls. Likewise, the pigmentation often appeared dark and diffuse, a condition commonly found in slightly inhibited worms. Another phenomenon resulting from methylene-blue treatment was that the reconstituted animals often appeared swollen and presented a blue, granulated appearance in the region of the gut.

It must also be remembered that 1/200,000–1/300,000 methylene blue will kill the pieces if they are allowed to remain in such a concentration much longer than 3 days. It has been pointed out that intact animals can survive from 5 to 7 days in 1/200,000 methylene blue but become heavily stained, inactive, and strongly insensitive before that time. Pieces become heavily stained, inactive, and insensitive by 3 days' exposure—phenomena which appear to result from an inhibitory, rather than a stimulating, action. Because of these and the foregoing observations it seems not unlikely that the increase in head frequency of pieces submitted to methylene blue for 72 hours may be due to an elimination or blocking of the factors inhibiting head formation which have their origin at the posterior cut surface.

With methylene blue increasing head frequency and with cyanide, in general, lowering head frequency, it might be expected that the two different agents acting together would bring about a head frequency somewhere between that which would appear when the two were used separately. In other words, these two agents might mutually antagonize each other in their effects on reconstitution. To some extent this prediction has been borne out, but it certainly does not hold in many cases. In stocks which show a low head frequency in the controls (Fig. 6), the combined solution determines a head frequency of pieces from *B*, *C*, and *D* levels which is above that when cyanide alone is used and below that of methylene blue (Fig. 6, curve *O*). In *A* pieces, however, the combined solution determines a head frequency that is definitely lower than with cyanide alone. In animals which show a high head frequency in the controls (Fig. 7) the combined solution produces a head frequency of *B*, *C*, and *D* pieces which is slightly below that of cyanide alone, while *A* pieces are apparently unaffected.

The interpretation of these data is not easy, and the following suggestions may not be entirely correct. If we assume, however, that the chief effect of cyanide is by direct action on the head-forming cells and the chief effect of methylene blue is in blocking or eliminating the posterior head-inhibiting factor, then we can offer at least a partial explanation. We have seen that, in all cases where methylene blue alone is used, the head frequency is increased at all levels with the exception of *A*, which already is producing the maximum. When cyanide is used with methylene blue, in all cases (with the exception of *A* pieces in animals with a high head frequency) the head frequency is considerably lower than with methylene blue alone. It has been noted that the cyanide–methylene-blue solution stains the pieces just as intensely (or apparently as intensely) as methylene blue alone. This being the case, there seems to be no reason why the methylene-blue complement of the cyanide–methylene-blue solution should not block or eliminate the posterior head-inhibiting factor. With the head-inhibiting factor blocked, the head-forming cells would be released and would tend to grow if it were not for the cyanide. With the cyanide present in the solution, however, the head-forming region, although released from the posterior inhibiting factor, is inhibited directly by the cyanide. Worms which show a low head frequency are in a different physiological state from those with a high head frequency. Apparently, when the head-forming regions of pieces with a high-control head frequency are partially released from the effect of posterior cut surface, the reaction to cyanide is more intense than with pieces which show a low head frequency, as might be expected if increased cellular activity results. Only with this assumption can we explain why *B*, *C*, and *D* pieces of stocks which show a high head frequency show a greater decrease in head frequency with the cyanide–methylene-blue solution than with the cyanide alone. As regards *A* pieces, it has been found that in stocks with a high head frequency these levels are not affected by either cyanide or cyanide–methylene-blue solutions (Fig. 7). In this case it may be suggested that, because of a higher activity of the level, *A* pieces are able to recover from the 72 hours' initial exposure. In stocks with a low-control head frequency, *A* pieces are slightly inhibited by cyanide; and with the cyanide–methylene-blue solution they are even more inhibited. Apparently, in this case a release of any posterior effect plus the direct effect of cyanide gives such an intense reaction that complete recovery does not occur.

The experiments in which the intact animals were submitted to cyanide for 6 days before sectioning and then allowed to reconstitute in methylene blue for 72 hours after section are of interest in that methylene blue restores, in part, the normal (control) head frequency in the cyanide-treated animals. Whether methylene blue in this case causes a direct increase in metabolism of cyanide-inhibited animals, a stimulation antagonizing the toxic effect of the cyanide, or an increase in head frequency by block or elimination of the posterior factor, can only be determined by further experiment.

SUMMARY

1. Pieces (one-eighth the length of the intact animal) from *B*, *C*, and *D* levels of 14–16-mm. worms, which under control conditions give a high head frequency, give a lowered head frequency when exposed to M/100,000 KCN for 72 hours after section.

2. In animals with a low-control head frequency in pieces from the posterior levels of the anterior zooid, exposure for 72 hours to M/100,000 KCN results in a lowered head frequency of pieces from *A*, *B*, and *C* levels but an increased head frequency of pieces from *D* level.

3. Methylene blue, in concentrations of 1/1,000,000 for the full reconstitutional period to 1/200,000 for 60 hours after section, caused an increase in head frequency of *B*, *C*, and *D* pieces in all experiments.

4. In animals which show a low head frequency in the controls, an exposure of 72 hours to a combined solution of 1/300,000 methylene blue plus M/100,000 KCN determines a head frequency of pieces from *B*, *C*, and *D* levels above that for cyanide and below that for methylene blue alone. This combination also lowers the head frequency of *A* pieces to a greater extent than does cyanide alone.

5. In animals which show a high head frequency in the controls the combined cyanide–methylene-blue solution determines a head frequency in pieces from *B*, *C*, and *D* levels which is below that for cyanide alone, while *A* pieces are apparently unaffected.

6. Animals (with a high-control head frequency) treated with M/100,000 KCN for 6 days, sectioned, and allowed to reconstitute in well water show a marked decrease in head frequency in pieces from *B*, *C*, and *D* levels. Similar animals, sectioned and allowed to reconstitute in 1/200,000 methylene blue for 72 hours, show an increase in head frequency over those allowed to reconstitute in well water.

7. The evidence indicates that the action of cyanide on reconstitution in *Euplanaria* is chiefly through an inhibition of the head-forming cells, while methylene blue probably acts chiefly by blocking or eliminating head-inhibiting factors which arise at the posterior cut surface.

LITERATURE CITED

BODINE, J. H., and BOELL, E. J. 1936. Effect of methylene blue on respiration of blocked and developing embryonic cells. Proc. Soc. Exper. Biol. and Med., 34:629.

———. 1937. The action of certain stimulating and inhibiting substances on the respiration of active and blocked eggs and isolated embryos. Physiol. Zoöl., 10:245.

BROOKS, M. M. 1932. The effect of methylene blue on HCN and CO poisoning. Amer. Jour. Physiol., 102:145.

———. 1933. The effect of respiratory poisons and methylene blue on cleavage of certain eggs. Collecting Net, 8:95.

BUCHANAN, J. W. 1922. The control of head formation in *Planaria* by means of anesthetics. Jour. Exper. Zoöl., 36:1.

CHILD, C. M. 1911*a*. Experimental control of morphogenesis in *Planaria*. Biol. Bull., 20:309.

———. 1911*b*. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. I. Jour. Exper. Zoöl., 10:265.

———. 1911*c*. Studies, etc. II. *Ibid.*, 11:187.

———. 1912. Studies, etc. IV. *Ibid.*, 13:103.

———. 1914*a*. Studies, etc. VII. *Ibid.*, 16:413.

———. 1914*b*. Studies, etc. VIII. *Ibid.*, 17:61.

———. 1916. Studies, etc. IX. *Ibid.*, 21:101.

———. 1920. Studies, etc. X. *Ibid.*, 30:403.

1924. Physiological foundations of behavior. New York: Henry Holt & Co.

CHILD, C. M., and WATANABE, Y. 1935. The head frequency gradient in *Euplanaria dorotocephala*. Physiol Zoöl., 8:1.

HINRICHS, M. A. 1924. A study on the physiological effects of caffein upon *Planaria dorotocephala*. Jour. Exper. Zoöl., 40:271.

HUG, H. 1932. La intoxicacion por el acido cianhedrico. II. Accion antidotica del azul de metileno, sulfuro de sodio y nitrito de sodio. Rev. Soc. Argentina Biol., 8:270.

MILLER, F. S. 1937. Some effects of strychnine on reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl., 10:276.

RULON, O. 1936. The effects of carbon dioxide, the hydrogen ion, calcium, and experimental conditioning on reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl., 9:170.

———. 1937. The effects of certain organic acids on reconstitution in *Euplanaria dorotocephala*. *Ibid.*, 10:180.

WATANABE, Y. 1935*a*. Rate of head development as indicated by time of appearance of eyes in the reconstitution of *Euplanaria dorotocephala*. Physiol. Zoöl., 8:41.

———. 1935*b*. Head frequency in *Euplanaria maculata* in relation to nervous system. *Ibid.*, p. 374.

STUDIES ON HETEROPLASTIC TRANSPLANTATION IN TRICLADS
I. CEPHALIC GRAFTS BETWEEN EUPLANARIA DOROTOCEPHALA AND E. TIGRINA[1]

(Ninety-eight figures)

JAMES A. MILLER
University of Chicago and Marine Biological Laboratory

IT IS commonly recognized by experimenters that by the production of abnormal relationships between parts it is often possible to isolate some of the factors which operate in the development and maintenance of the normal characteristics of organisms. The large volume of work on extirpation, explantation, and transplantation is evidence of the possibilities of this type of experimental procedure.

In the study reported here, interspecific transplantation among triclad flatworms was selected as a method of studying certain phases of "dominance," a phenomenon which has been recognized in the zoölogy laboratory of the University of Chicago for the last quarter of a century (Child, 1911*a*) and has recently come to the attention of embryologists in various parts of the world (Huxley and de Beer, 1934; Huxley, 1935; Weiss, 1935; Strelin and Trifonova, 1935). For a brief review of the characteristics of the dominant region of the simpler invertebrates see J. A. Miller (1937).

Most of the earlier workers on transplantation in the lower invertebrates have been concerned with problems other than those attacked here. However, numerous instances of dominance may be gleaned from their data. Among coelenterates, Browne (1909) found that small pieces of the hypostome region of *Hydra* will determine a new apical region in the host and modify its polarity. Rand, Bovard, and Minnich (1926) confirmed these results and demonstrated the "inhibitory dominance" of such a graft. Mutz (1930), using *Pelmatohydra* and *Hydra*, showed that the effects are not species specific. Burt (1934) found a gradient in the ability of grafted rings of tissue from different levels of *Pelmatohydra oligactis* to dominate the host. Child (1929*b*, 1932) studied dominance in *Corymorpha* by means of grafting. In this form pieces from all levels may induce a new hydranth and stalk, but distal pieces are most potent. It is not even necessary to implant any tissue, for laceration of the edges of an incision is sufficient stimulus to cause the cells in the vicinity to produce a hydranth (Child, 1927, 1929*b*).

Among the flatworms, Moretti (1911), using *Planaria torva*, found that only grafts of the head could persist and maintain the autonomic movements. He figured and described cases of inhibition of regeneration after removal of the host head and of induction of a pharynx by grafts of the cephalic region but did not discuss them. Rand and Browne (1926) reported four cases in *Euplanaria tigrina* (syn. *Planaria maculata* [Kenk, 1935]) in which there was no regeneration of the host head after its removal close to a cephalic graft. Gebhardt (1926) reported a few cases in *Planaria lugubris* in which there were outgrowths of the body as a result of grafting. Goetsch (1926, 1929) found that a graft of a regenerating head could prevent the development of a host head and eventually take its place. When a small portion of the host was isolated with the graft, it not only prevented development of head and tail but seemed to grow at the expense of the host. Santos (1929, 1931) has made the most complete study of dominance by the grafting technique. He found that ganglionic grafts of *Euplanaria dorotocephala* in prepharyngeal regions could induce the development of postcephalic structures. In pharyngeal regions they might induce a pharynx, and in postpharyngeal regions two pharynges might be induced with the one anterior to the graft in reversed polarity. The indications were that the ability of the graft to inhibit the regeneration of a host head depends upon the level of implantation. These effects were not species-specific, for heteroplastic transplants between *E. dorotocephala* and *E. tigrina* gave the same results. Grafts other than from the head did not possess this reorganizing power.

---

[1] The writer wishes to express very great appreciation for the interest and inspiration of Professor C. M. Child in this work, as well as for proposing this line of investigation and for helpful suggestions during its progress.

Steinmann (1933), using *Dendrocoelum lacteum*, reported a case in which a ganglionic graft placed in the tail developed two small pharynges and more or less complete sets of reproductive organs. Finally, Okada and Sugino (1934) reported cases of dominance of host by graft in the Japanese *Planaria gonocephala*, in which not only pieces containing the cephalic ganglia but also those from between the head and pharynx could induce pharynges in postpharyngeal regions of the host.

In the experiments reported here heteroplastic transplants were used as a means of distinguishing between implant and host in order to secure more exact data on the regeneration of the graft, the ability of grafts from various levels of the donor to maintain themselves, the differences in inductive capacity of grafts from various levels, and the differences in resistance of various host levels to the domination of grafts.

MATERIAL

The animals used in these experiments were *E. dorotocephala*, collected in spring-fed streams near Rockford and Cary, Illinois, and Valparaiso, Indiana; *E. tigrina* (Kenk, 1935; syn. *P. maculata*) from Valparaiso; and *E. tigrina novangliae*[2] from a pond in Falmouth, Massachusetts. These three forms are closely related anatomically and proved well adapted to the requirements of the experiments reported here. Takes were readily secured; yet there were sufficient external differences to distinguish between graft and host tissues, a fact which permitted accurate data on the fate of the graft and on its inductive capacity. Most of the transplantations were between *E. dorotocephala* and *E. tigrina novangliae*.

The external differences by which these three forms may be distinguished are listed below. They consist of differences in size, shape, pigmentation, and appearance of the digestive tract and of the photoreceptors. *Euplanaria dorotocephala* (Pl. I, Fig. 1) is the longest of the three; *E. tigrina* (Pl. I, Fig. 2), the shortest; and *E. tigrina novangliae* (Pl. I, Fig. 3), the widest. There are consistent differences in shape of head. The head of *E. dorotocephala* is pointed with long "auricles" and a slight constriction in the "neck region"; that of *E. tigrina* is blunt with short and very wide "auricles" and with very little constriction in the "neck region."

With respect to pigmentation the three forms are quite distinct. The pigment of *E. dorotocephala*, almost black in nature and dark to light brown when kept in the laboratory, is distributed over the surface of the animals in small, dark granules against a brown or tan background. The ventral surface is a grayish hue because of the lighter background than that on the dorsal surface. The dorsal surface of *E. tigrina* (as represented at Valparaiso, Indiana) is finely maculated with yellowish-orange pigmented areas against a tan background. The ventral surface of this form is white with gray pigment areas, giving a light-gray effect. The coloration of *E. tigrina novangliae* is very variable; therefore, only one type was used in these experiments. The dorsal surface of this type is characterized by large pigmented areas, some of which are light and some of which are dark brown, interspersed with occasional colorless areas, and by a mid-dorsal light stripe, along each side of which there is an irregular row of darker spots. The ventral surface of these planarians is much darker than that of *E. tigrina maculata* and has a brownish tinge.

---

[2] *Euplanaria tigrina* and *E. tigrina novangliae* are so closely related that Kenk (1935, p. 85) stated that "the copulatory organs afforded no means of discriminating between them" but that on the basis of the external differences they may possibly be subspecies.

The photoreceptors of the three can be distinguished by the differences in the size and shape of the unpigmented areas. Those of *E. dorotocephala* are elongated and set in such a fashion that the anterior parts are closer together than the posterior. Those of *E tigrina* are very large and are placed with their axes parallel to each other. The crescentic pigmented areas of the photoreceptors are completely visible in this form. In the case of *E. tigrina novangliae* the unpigmented areas are less perfectly oval, are smaller, and do not completely expose the pigmented areas.

The branches of the digestive tract of living planarians may be readily observed by allowing the animals to feed on clotted or dried blood. Those of *E. dorotocephala* are characterized by extensive anastomoses and by the formation of accessory longitudinal trunks in connection with both anterior and posterior rami of the intestine (Hyman, 1925). On the other hand, the branches of the intestine of *E. tigrina* are more regular and do not develop the anastomoses and accessory trunks such as are characteristic of the other species.

Preliminary transplants were made according to the method described by Santos (1931); however, it was found that the degree of anesthesia could be more easily controlled and that recovery was more rapid when a physical agent (low temperature) was used instead of a chemical one (chloretone). Likewise, it became apparent that the use of rectangular grafts containing the same volume of tissue as the triangular ones used by Santos resulted in a higher percentage of "takes," since incisions of this shape had less tendency to cause the host to tear apart at the angles. With these modifications, very high percentages of unions were obtained, usually between 75 and 100 per cent.

PROCEDURE

Except in cases in which grafts were made in the cephalic region the prospective hosts were decapitated from 2 to 4 hours before operation in order to reduce their activity. The recipient was placed, together with a small amount of well water, on a clean, grease-free glass square resting on a pan of ice from a refrigerator. When the host was sufficiently anesthetized, a rectangular window was removed with the aid of two very sharp half-spear dissecting needles under a low-power (55-mm. objective) binocular microscope. Because of the strong contraction of the cut surfaces, it was found necessary to make the graft considerably smaller than the window into which it was to be placed. It was placed in the desired orientation, and the excess water drained off the plate. Then a Petrie dish with vaselined edges was inverted over the plate, making an airtight chamber. This was placed in the dark for 8–12 hours, after which the "takes" were transferred to finger bowls with approximately 200 cc. of well water, and were measured and recorded. At this time controls from the same stock were cut with windows as nearly like those of experimentals as possible, but no graft was inserted. Both experimental and control animals were fed beef liver or dried blood, and the water was changed twice a week. Subsequent observations were made at weekly intervals. Certain of the specimens were killed in a 0.5 per cent solution of HNO_3 and fixed in a saturated solution of $HgCl_2$ in a 0.6 per cent solution of NaCl (Willier, Hyman, and Rifenburgh, 1925). A report of the histological study of these grafts will be made at a future date.

Large specimens of the three forms were used. Those of *E. dorotocephala* were between 17 and 22 mm. in length; those of *E. tigrina* between 11 and 14 mm.; and those of *E. tigrina novangliae* between 11 and 16 mm. The grafts usually ranged in size from 0.8 × 1.0 mm. to 1.1 × 1.3 mm. The few that were smaller occasionally were resorbed, while in larger ones the frequency of takes was low.

Three regions of the body were selected for study: the head region, which contains a concentration of nervous elements; the pharynx, which contains highly differentiated muscular and nervous elements; and the tail, which is the actively growing but least differentiated portion of the animal. Grafts were taken from each of these regions of the donor and transplanted into each of three regions of the hosts. All possible combinations of dorsal and ventral with anterior and posterior surfaces of graft and host were made.

In order to make the results comparable, grafts were cut from, and implanted into, as nearly the same levels as possible. All cephalic grafts and windows in the hosts were cut with the anterior surface just in front of the unpigmented areas of the eyes; pharyngeal grafts and windows were cut to include the muscles attaching the proximal end of the pharynx to the body wall. Tail grafts and windows were cut from the most posterior tips of the tail. They were made slightly wider at the anterior end to include as much of the growing region as possible. In addition, 20 cephalic grafts were implanted just behind the eyes for comparative purposes. In this report only the results of grafts from the cephalic region will be discussed.

Since neither the head nor tail levels of this study corresponded exactly to the regions used by Santos (1929, 1931), a different terminology has been employed in referring to them. The head grafts in this study did not contain the entire ganglionic region and therefore are called "cephalic," rather than "ganglionic," grafts. Similarly, the most posterior level used is designated as the "tail" region, and grafts from it as "tail grafts," because they were located more posteriorly than the postpharyngeal level of Santos. The "tail" represents the more posterior levels of the posterior zooid region. The terminology used in describing the orientation of the grafts is a modification of that introduced by Harrison (1921) and widely used in amphibian experiments. When the dorsoventral axes of graft and host coincided, the implant is referred to as a "dorsodorsal"; when reversed, it is called a "dorsoventral." When the anteroposterior axes coincided, the graft is an "anteroanterior"; when reversed, an "anteroposterior."

Records were kept on mimeographed sheets with columns for date, measurements (graft length and width; host length and width), drawings, and notes. The notes consisted of observations on the extent of union between graft and host, the development of the transplant, the behavior of host and graft, and feeding responses. In the more recent experiments the weekly drawings were supplemented by photographs taken with a Leica camera and a 1:1 copying attachment. In most cases the outlines of the digestive tract were recorded after feeding the specimens dried blood.

EXPERIMENTAL

GENERAL OBSERVATIONS ON GRAFTS AND CONTROLS

A total of 412 cases of successful transplantation of cephalic pieces were studied. Of these, 105 were placed in the head region, 20 in the anterior prepharyngeal region, 113 in the pharyngeal region, and 174 in the tail. No species differences in inductive potency of grafts or in resistance to induction by the host, as indicated by Santos (1931), were noted. For this reason, and in order to avoid excessive length in the experimental section, no distinctions between species will be made in the descriptions.

Takes.—Although in all types of transplants reported here 100 per cent takes were obtained upon occasions, in general union occurred more commonly with *E. tigrina* as host than with *E. dorotocephala.* Of the three host regions employed, the tail gave the lowest percentage of takes and the head the highest. Of the four cut surfaces of the graft, the anterior united with the host the least frequently and the posterior the most.

Behavior.—Implants in the head in dorsodorsal anteroanterior orientation which united by all surfaces often became immediately incorporated into the host, and normal behavior of the host became re-established within 12 hours after operation. When possessing a free surface or placed in other orientations, grafts in the head usually maintained their own individuality but did not extend their dominance appreciably into the surrounding tissue. On the other hand, when placed in the pharynx region, cephalic grafts controlled the movements of more posterior portions of the host but were unable to gain ascendancy over the more anterior regions. The orientation and degree of union did not greatly affect the ability of grafts to control host movements when placed in the pharynx or tail. Cephalic grafts placed in the tail region controlled the movements of the host both anterior and posterior to the level of implantation.

When two or more heads were present in the same animal, as was the case in most of the experimental animals, considerable difficulty was experienced in inducing the worms to feed. The stimulation by food was considerably greater in these than in normal worms; and, though they would crawl over the food again and again, the pharynx was unable to start feeding before being dragged off by the very active heads. In some cases it was possible to induce these animals to feed by covering the containers with a black cloth. In the absence of stimulation of the photoreceptors the activity of the heads was sufficiently reduced to permit the institution of feeding-responses by the pharynx.

Regeneration.—In all levels a free surface was necessary for regeneration of the normal head shape by the implant. The free surface likewise determined its polarity, with modifications up to 180° not uncommon.

Union of the digestive tracts.—Union of the intestinal diverticula of the graft with those of the host was necessary for the persistence, as well as for the growth, of the graft. However, even in starving grafts, regeneration of lost parts from a free surface occurred, and the ability to induce host structures was not appreciably reduced.

Disintegration of host tissues in the neighborhood of implants.—In certain grafts in the head, prepharyngeal, and pharyngeal regions disintegration of tissue anterior to the level of implantation occurred. In most of these cases no food was observed, after feedings, in the regions where disintegration occurred later. In the rest, the digestive tracts in the affected regions reunited with small side branches and often received little or no food. In all, the typical changes which have been observed in starving planarians (including disintegration of the head) were noted. Accordingly, it is suggested that the disintegration of the heads in these cases might have been caused by starvation.

In many of the cases in which union of graft and host was only partial, sooner or later the host tissue on one side of the graft disintegrated and the implant which had projected dorsally from the host came to lie in the same plane as the host. Disintegration of this localized area is thought to have occurred as a result of the activities of the graft, which, in applying itself to the substrate, constricted the host tissue lateral to it. Whether disintegration was due to mere mechanical pressure or to some secondary effect of the constriction, such as interference with food supply and waste-removal, cannot be said.

Controls.—In the controls, in which a window was made but no graft was inserted, the cut surfaces united within a few hours after the operation. In the head the anterior and posterior surfaces usually united, while in the pharyngeal and tail regions it was the lateral surfaces of the windows which fused. Usually within 2 weeks new tissue had filled in the injury completely, and the structures which had been removed were replaced. There were very few exceptions to this procedure; they will be treated in their appropriate places in the sections which follow.

GRAFTS IN THE HEAD REGION (105 CASES)

Dorsodorsal anteroanterior orientation of graft (24 cases).—The behavior of cephalic grafts implanted in the head region depended principally upon their orientation and the degree of union with the host. When both dorsoventral and anteroposterior axes coincided, grafts and hosts united along all surfaces (22 of 24 cases). Within 12 hours after operation the experimental animals were observed to crawl normally and react normally to food; whereas in the case of the controls, normal locomotion and feeding responses did not appear until regeneration had proceeded as far as the development of approximately the normal head shape and photoreceptors, a week or more later. In almost all cases the photoreceptors of the implants happened to lie in the eye-level of the host. In these, proliferation of host tissue was at a minimum and no host eyes[3] were reconstituted (Figs. 4, 72). In contrast to the experimentals the control planarians in every case regenerated the tissue removed, including the two photoreceptors. In 3 cases supernumerary eyes were developed (Figs. 5, 6).

When the photoreceptors of the implants did not happen to lie in the eye-level of the host, one or more eyes were reconstituted by the host (Fig. 7).

In 4 cases the tip of the host head disintegrated, leaving the anterior portion of the graft exposed. These resulted in a chimaera-type head of which the mesial part was of one species and the "auricles" and sides were of the other (Fig. 8).

In all grafts which were kept 3 months or more, there was a gradual replacement of graft epidermis and of graft photoreceptors by those of the host. Whether other tissues were involved is not known. This replacement, which was observed not only in grafts in the head region but also in those in the other two levels used in this study, proceeded as follows. Usually within 1 week after implantation the edges of the transplant became less clearly defined and a ring of unpigmented tissue was observed surrounding the graft. This ring of new tissue (regenerating tissue in these planarians does not become heavily pigmented for 2 weeks or more) was observed gradually to encroach upon the graft during the next 6–8 weeks until all that could be seen of the implant was a few darker pigment spots between the photoreceptors. Eventually they, too, disappeared. Meanwhile, pigment spots characteristic of the host species developed around the periphery of the new tissue, showing that the cells replacing those of the graft were of host and not of graft origin. It was not possible to follow the process of replacement of the photoreceptors with the same degree of assurance as in the case of the epidermis. However, the change in shape of the unpigmented areas from that characteristic of the graft to that of the host seemed to occur following the depigmentation of the surrounding tissue. The black crescentic pigment areas of the photoreceptors also changed shape gradually but by such imperceptible stages that in most cases the process could not be followed. In 3 cases the black pigment areas of the photoreceptors of the graft were observed to decrease in size gradually, and later a new pigment spot appeared in the unpigmented area of each eye. This increased in size until it fused with that of the graft. Whether this represents the usual situation is not known.

---

[3] Strictly speaking, planarians possess photoreceptors and not eyes, since they have no apparatus for focusing an image upon the photosensitive cells. However, in this report "eye" is used in its less exact sense as a synonym of photoreceptor.

GRAFTS IN THE HEAD REGION

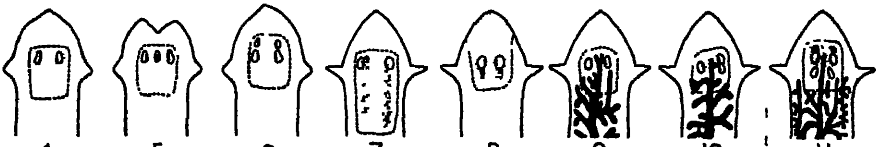


FIG. 4.—*E. dorotocephala* into *E. tigrina novangliae*. DDAA.[*] Union complete. 31 days. Epithelia of implant were eventually replaced by host tissue.

FIGS. 5 AND 6.—*E. tigrina novangliae*. Controls 31 days. FIG. 5.—Three photoreceptors have developed in place of the two which were removed. FIG. 6.—Four photoreceptors have developed in the new tissue.

FIGS. 7 AND 8.—*E. tigrina novangliae* into *E. dorotocephala*. DDAA. 35 days. FIG. 7.—Union complete. Large graft (1.4 × 0.8 mm.). Eye appeared in new tissue left of implant 2 weeks after grafting. Left graft eye became reduced in size and subsequently fused with it. FIG. 8.—Anterior edge of implant failed to unite with host. Host head disintegrated anterior to implant, resulting in chimaera-type head.

FIGS. 9, 10, AND 11.—*E. tigrina novangliae* into *E. dorotocephala*. DDAP. Union complete. FIG. 9.—35 days. A small photoreceptor appeared anterior to right graft eye 1 week after implantation and disappeared 2 weeks later. Graft epithelia completely replaced by new tissue. Note that digestive tract extends farther anteriorly than normally. FIG. 10.—14 days. One eye appeared posterior to left graft eye 1 week after implantation. Four weeks later new tissue had completely replaced graft epithelia. FIG. 11.—28 days after operation. Two eyes have developed in new tissue posterior to implant.

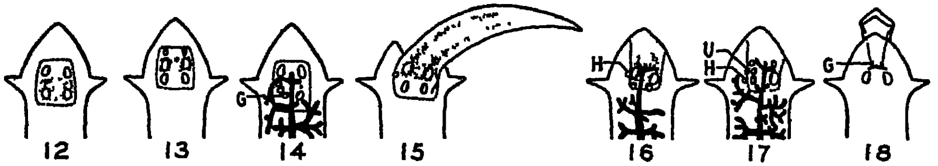
Dorsodorsal anteroposterior orientation of graft (64 cases).—Cephalic transplants placed in the head region with their anteroposterior axes reversed were some of the most interesting of all the types studied. The percentage of cases of complete union (42 per cent) was less in this class of grafts than in those in which the anteroposterior axes coincided. When reversed, the relatively thick posterior surface of the graft was in contact with the thin anterior surface of the host window, and vice versa. It is thought that this was chiefly responsible for the proportionally fewer cases of complete union in these grafts than in the class just reported.

In cases of complete union the grafts showed little independent activity, and the proliferation of tissue resulting from the implantation was so slight that in 5 cases no outgrowth was noted and in the rest it was very small. In all but 4 cases more than one photoreceptor developed. Of the exceptional cases, 1 developed a very small eye which later regressed (Fig. 9), and the other 3 cases regulated a permanent photoreceptor (Fig. 10). The usual result was the development of one or more photoreceptors in the new host tissue either anterior or posterior to each eye of the graft. In 3 cases one supernumerary eye developed anterior to each eye of the graft, and in 3 two eyes developed posterior to those of the graft (Figs. 11 and 12). There were 3 cases in which four eyes developed, two of them anterior and two posterior to those of the implant (Fig. 13). In other cases the supernumerary photoreceptors were not as regularly arranged, although they never appeared very far from the region of the mesiolateral axis at which eyes are found normally (Fig. 14). It was noted that the supernumerary eyes usually appeared considerably later than did the regenerating eyes of the controls, with a delay of 2 or 3 weeks not uncommon.

---

[*] In these and subsequent figures the following conventions are used: graft tissue stippled where distinguishable, approximate extent of new tissue indicated by broken lines, invaginated portions by alternating dots and dashes, main intestinal branches by solid black. Time after operation given in days. Orientation of implants: DDAA = dorsodorsal, anteroanterior; DDAP = dorsodorsal, anteroposterior; DVAA = dorsoventral, anteroanterior; DVAP = dorsoventral, anteroposterior.

GRAFTS IN THE HEAD REGION



FIGS. 12, 14, 15, 16, AND 17.—*E. tigrina* into *E. dorotocephala*. FIGS. 13 AND 18.—*E. tigrina novangliae* into *E. dorotocephala*. In all but Figs. 15 and 18 union was complete. DDAP.

FIG. 12.—35 days. Two eyes have developed in new tissue posterior to implant.

FIG. 13.—28 days. Two eyes have developed in new tissue anterior to implant and two more posterior.

FIG. 14.—56 days. Two eyes appeared anterior to implant 1 week after implantation. Three more developed later in new tissue between graft and host eyes. Left graft eye (G) has become reduced in size.

FIG. 15.—Host disintegrated anterior to implant. 42 days. Tail-like outgrowth has regenerated from free graft surface. Two eyes developed in new tissue opposite graft eyes 1 week after implantation. Unpigmented area on left and projection on right developed later.

FIGS. 16 AND 17.—7 and 49 days, respectively. FIG. 16.—Unpigmented portion of left host eye (*H*) not completely removed. Two eyes have appeared posterior to graft. FIG. 17.—Graft epithelia completely replaced by new tissue. Graft eyes have fused with two supernumerary eyes shown in Fig. 16. Five additional eyes developed anterior to graft eyes but have been reduced by fusions to three. Unpigmented projection (*U*) had developed to left of implant.

FIG. 18.—35 days. Posterior surface of graft failed to unite with host. Resulting tubular outgrowth lined with ventral epithelium, projects from ventral surface of host. Pigment areas of graft eyes (*G*) visible. Two eyes present in new tissue posterior to implant.

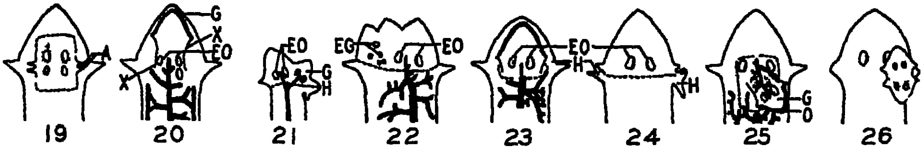
A comparison of the conditions of the digestive tracts of the experimental and control animals showed that in the former, as a result of reversing the anteroposterior axis of the graft, the intestine extended into the head of the compound well anterior to the photoreceptors of the implant. In the controls the most anterior branch ended between the regenerated eyes. Usually when there were side branches of the digestive tract anterior to the eyes of the graft, supernumerary eyes developed anterior to those of the implant. However, the correlation between diverticula and number of eyes was not sufficiently close to be interpreted as the determination of the latter by the former. It seems probable that farther anterior extension of the digestive tract in these specimens merely furnishes a greater supply of nourishment to the more anterior levels of the head and allows the development of eyes which might otherwise suffer from competition with the graft.

The presence of a free surface in cases of incomplete union resulted in greater activity on the part of the implant and considerably greater proliferation of tissue as compared with cases of complete union. In 3 cases the host tissue anterior to the implant disintegrated within a day or two after grafting, leaving the posterior surface of the graft free. In two of these the implant retained its original polarity and showed almost continuous activity, contracting and extending entirely independently of the movement of the host. An outgrowth resembling a tail developed from the free surface of the implant, and two eyes formed in the regenerating host tissue directly behind and opposite those of the graft (Fig. 15). In the third case fusion of graft and host occurred without any appreciable proliferation of new tissue, as was the case in grafts in which both dorsoventral and anteroposterior axes coincided. However, within a week two supernumerary eyes had appeared posterior to those of the implant (Fig. 16). During the next 3 weeks five more developed anterior to them, and in addition there was by this time an unpigmented projection suggestive of an abortive cephalic lobe to the left of the graft. In the ensuing 4 weeks the eyes of the graft fused with the two supernumerary eyes that were posterior to them, and fusions were occurring in the eyes which had developed anterior to the graft (Fig. 17). Thus, even though the implant had its polarity reversed as a result of its position, nevertheless it produced a marked effect upon the host head.

In 19 cases a part of one surface of the graft failed to unite with the host. From this free surface and the cut surface of the host developed a tubular outgrowth around which later appeared supernumerary eyes. Usually it was possible to determine by the position of the developing eyes that the free surface was anterior, even though in some cases it developed from the lateral or posterior surface of the graft. In most cases the outgrowth extended from the upper surface of the host and was surrounded by dorsal epithelium with the ventral surface on the inside of the tube. In one case, however, it extended ventrally and the dorsal surface was inside (Fig. 18). Figure 19 shows a case in which there were two free surfaces—one a small portion of the left, and the other a smaller portion of the right side of the implant. The two free surfaces developed outgrowths resembling cephalic lobes with their unpigmented sensory areas, and a second pair of photoreceptors developed in the regenerating host tissue opposite the anterior surface of the graft.

In 8 cases the entire anterior surface of the implant remained free. In these the anteroposterior axes of the transplants were not affected. Each graft regenerated a normal or nearly normal head, and the host developed a second head from the free edge of the window directly opposite the graft. This grew rapidly until it reached the size of the head of the implant, and during all subsequent observations both remained the same size except when conditions were altered. The presence in the ganglionic region of the host of an *active* graft with its anteroposterior axis reversed was sufficient, in all cases except one, to inhibit completely the development of eyes anterior to it. It appears that grafts that were able to escape the domination of the host and retain their own individuality, because of the free anterior surface, so altered conditions in the head of the host that the physiological level at which eyes are produced did not develop. On the other hand, it should be noted that, even when an implant lacking a free surface became dominated by the host to the extent of having its polarity reversed, it still exerted a modifying influence on the host, extending the physiological level at which eyes could develop over a much wider area than is normally found in these species. In this connection it might be recalled that Abeloos (1930) reports finding specimens in his stocks of *P. gonocephala* with supernumerary eyes usually arranged, as was the case in these experiments, in pairs extending toward the anterior margin of the head. He listed other reports of supernumerary eyes (Janichen, 1897; Lang, 1913) and attributed them to senescent conditions, since they usually appeared in the largest specimens. However, Child (1921) has shown that susceptibility to inhibiting agents acting during head regeneration, as indicated by modifications of head-form and position or absence of eyes, decreases from the median region laterally. This indicates a higher gradient level in median than in lateral regions. Consequently, the hypothesis that the degenerative changes associated with senescence should cause an increase in the breadth of the area in which eyes are found seems improbable. In the present studies it was noted repeatedly that, when supernumerary eyes appeared, they developed in the new tissue during the period of active regeneration of the host, and that later there was a tendency for the eyes to regress and be resorbed.

GRAFTS IN THE HEAD REGION



FIGS. 19–22.—*E. tigrina* into *E. dorotocephala*. FIGS. 23–25.—*E. tigrina novangliae* into *E. dorotocephala*. FIG. 26.—*E. dorotocephala* into *E. tigrina novangliae*. In all but Figs. 19 and 26 anterior surface of graft failed to unite with host. Orientation of all DDAP.

FIG. 19.—21 days. From each lateral free surface two auricle-like structures (*A*) developed. Two eyes have appeared posterior to graft.

FIGS. 20 AND 21.—28 and 56 days, respectively. FIG. 20.—Tubular outgrowth, of which the graft forms the anterior half, has developed. Three eyes illustrated (*EO*) are in host portion of outgrowth. Ventral surface of implant (*G*) visible just behind anterior edge of outgrowth. Disintegration of anterior part of host to *X–X* and of region of union between two components of tubular outgrowth resulted in condition shown in Fig. 21. Right remnant of host head (*H*) and graft (*G*) form right component, and host portion of outgrowth forms the other. Unpigmented areas of right eyes have fused. Graft epithelia have been completely replaced by host tissue.

FIG. 22.—28 days. Tubular outgrowth with two eyes (*EO*) developed and, by disintegration of more anterior parts of host and region of union on left, produced a wide, flat composite head. Double unpigmented area appeared at line of junction between graft and host. Eye has appeared beside right graft eye. Graft epithelia completely replaced by new tissue.

FIGS. 23 AND 24.—7 and 28 days, respectively. FIG. 23.—Graft (*G*) almost entirely hidden behind tubular outgrowth of new tissue in which are two eyes (*EO*). Digestive tract did not reach host or graft head. FIG. 24.—Host and graft heads disintegrated (host by fourteenth day after implantation, graft by twenty-first day), leaving only host portion of outgrowth and auricles of original head (*H*).

FIG. 25.—14 days. Host portion of tubular outgrowth (*O*) lies behind graft (*G*). In contrast to Fig. 23 graft and host digestive tracts united. During 5 weeks following, before it was fixed, graft and host portions of outgrowth continued to increase in size.

FIG. 26.—Graft united to host by posterior part of ventral surface only. 14 days. Digestive tracts of graft and host failed to unite. Head with reversed polarity developed from posterior edge of graft. Graft decreased in size rapidly and separated from host 3 or 4 days after this drawing was made.

In 3 cases the portion of the host anterior to the graft was devoid of intestinal branches. During 4 or 5 weeks these portions gradually decreased in size, lost their pigmentation, and finally disintegrated. The graft head and that of the host which had developed behind the graft now became the functional heads of the compound (Figs. 20 and 21). In 2 of the 3 cases the digestive tract of the graft did not unite with the host, and in these cases the graft also regressed. In 1 case it became an appendage on the left side of the second host head (Fig. 22); while in the other it, too, disintegrated, leaving the second host head at the anterior end of the host (Figs. 23 and 24).

Figure 25 shows the result when both the graft and the anterior portion of the host re-established connections with diverticula from the host intestine. In this case the graft and the head behind it grew until attaining a size practically equivalent to that of the original host head. A postcephalic outgrowth of tissue similar to that described by Santos (1931) was induced by both the graft and the host head.

In 2 instances the amount of union between implant and host was very slight. In both, even though the grafts rapidly decreased in size and gave evidence of starvation from the first, they regenerated "auricles" and the triangular head shape which is normal for the species used. In 1 case (Fig. 26) the graft developed a second head on its free posterior surface, reminding one of the findings of Morgan (1903) and Child (1911*b*) that very short transverse sections of planarians sometimes develop bipolar forms.

Dorsoventral anteroanterior orientation of graft (16 cases).—When transplants were made in the head region with the dorsoventral axis reversed, they usually united with the host by all four cut surfaces. Within 12 hours after implantation the compounds were actively crawling. However, in contrast to the first described grafts, the anterior ends of these were continuously raised above the substrate as if the ventral longitudinal muscles of the graft were in a state of contraction.

In 5 cases the anterior tip of the host disintegrated within the first week after implantation. The presence of the graft prevented the regeneration of the host, and the implant itself reproduced the missing parts from its anterior surface (Fig. 27 and Pl. I, Fig. 28).

In all cases in which the dorsoventral axis was reversed, there was some proliferation of tissue between the graft and the host. In 4 cases both graft and host were involved, with the resulting development of one or two additional heads. The dorsal one was composed of the dorsal surface of the host and the ventral surface of the graft, and the ventral one, below the host head, consisted of the dorsal surface of the graft and ventral surface of the host. In 2 cases the ventral supernumerary head was bipolar.

In two cases the graft and host united by the dorsal surfaces only. In these the graft regenerated an anterior end, auricles, a functional pharynx, and a tail. During the 10 months that they were under observation the grafts, which showed greater muscular activity than their hosts, likewise showed relatively greater growth. By the end of the period, the two components were approximately the same size and crawled equally well whether the host ventral surface or that of the graft was in contact with the substrate (Pl. I, Fig. 29). After feeding them blood, it was possible to determine that the digestive tracts of graft and host were anastomosed, but behavior tests indicated that there were no extensive connections between the nervous systems of the components. A remarkable feature of these examples—one by which they differed from the others of this series—was that the grafts retained their specific characters during the entire period of observation. This condition will be treated in the discussion.

Dorsoventral anteroposterior orientation of graft (11 cases).—Only a few grafts were made with their dorsoventral and anteroposterior axes reversed. As was true in the other cases of inversion of the dorsoventral axis of the graft, there was a tendency for the anterior edge of the implant to fail to unite with the host. In only 3 instances were there complete takes. These resembled those of the series mentioned above in their behavior, crawling with their heads continuously raised from the substrate. There was more or less proliferation of tissue between the graft and host, and in 1 case this was sufficient to inclose the graft completely. In this animal, eyes regenerated in the host and the graft regressed until it became a small projection on the anterior part of the host head. In the others the graft remained and no host eyes developed.

Incomplete takes usually failed to establish connections with the digestive tract of the host. Although they regenerated a more or less normal head, and in one instance a tail as well, they rapidly decreased in size and did not prevent the regeneration of a head by the host in cases in which it had previously disintegrated. In one incomplete take the digestive tract of the implant united with that of the host and the graft completely prevented any regeneration at the anterior end of the host (Pl. I, Fig. 30). In these, as well as in other dorsoventral grafts, there was no indication that the dorsal or ventral epithelia were modifiable.

Grafts in the anterior prepharyngeal region (*20 cases*).—Since the head contains the enlarged ganglionic part of the nervous system, there is the possibility that the resistance to induction at this level is a function of the amount of nervous tissue present. Accordingly, 20 grafts were implanted just behind the head. Not all possible combinations of dorsal and ventral with anterior and posterior surfaces were used, but the results were sufficient to show that there was no marked reduction in resistance in this level.

When grafts in normal orientation united completely with their hosts, there was no proliferation of tissue; and the only indication of induction was the appearance of a projection at each side of the implanted eyes (Pl. I, Fig. 31).

Figures 32 (Pl. I) and 33 show cases in which the grafts in normal orientation united by their posterior and a part of their lateral surfaces. A small postcephalic outgrowth developed in each case and subsequently tore through the right side of the host. The two differ in that in Figure 32 the graft and host heads are approximately equivalent, whereas in Figure 33 the host head has become reduced in size as a result of insufficient food reaching it from the branches of the digestive tract.

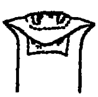
In Figure 34 we see a perfect take in which the anteroposterior axis was reversed. There was a slight proliferation of tissue around the graft, but otherwise no modification of the structure of the host was observed. Implanting the graft in reversed dorsoventral polarity usually resulted in union with the dorsal surface only. The grafts regenerated in nearly normal shape and induced a very small outgrowth of tissue (Figs. 35 and 36), producing a structure which resembled more or less several of Santos' (1931) figures (Figs. 4, 5, 6, 9, 13, and 14). There were two complete takes of dorsoventrally reversed grafts. In both of these a head developed composed of dorsal surface of the host and ventral surface of the graft (Fig. 37). In one the head developed from the anterior edge of the window and posterior edge of the graft, and in the other vice versa. In none of the grafts in this level was there any marked reduction of host resistance to reorganization. Accordingly, it may be concluded that the differential from anterior to posterior in resistance to induction is not dependent upon the presence or absence of certain structures concentrated in the most anterior levels, such as the cephalic ganglia.

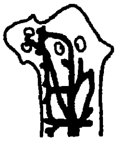
GRAFTS IN THE PHARYNGEAL REGION (113 CASES)

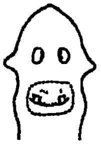
When a cephalic graft was implanted into the pharyngeal region, the regenerating pharynx appeared some distance posterior to the original location, whereas that of the controls developed in the new tissue which marked its original position. Since the pharynges of the experimental animals developed out of their normal locus, they may be designated as structures induced by the grafts. In contrast to those placed in the head, grafts in the pharynx induced the development of postcephalic outgrowths even in cases of complete union with the host. However, decapitation of the host at varying distances from the graft gave little evidence of graft dominance more than 2 mm. anterior to the level of implantation. Because of removal of the pharynges of the hosts, usually 3 or more weeks elapsed between the operation and the first feeding. During this time a tendency for the grafts to migrate was noted; and, accordingly, in certain instances longer periods of starvation were instituted, and cases of fusion of grafts with the host heads resulted. In both the pharynx and the tail regions the orientation of the implant had far less effect upon the final result than did the presence or absence of a free surface. For this reason the data in these sections will be described under two headings only.

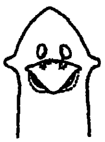
GRAFTS IN THE HEAD REGION

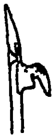
E. dorotocephala into *E. tigrina.*


27


33


34


35


36

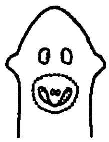

37

FIG. 27.—DVAA. Union complete. 7 days. New tissue around ventral surface of implant. Crawled with anterior portion of head raised. (Pl. I, FIG. 28.—Photograph of same animal 266 days later.)

GRAFTS IN THE ANTERIOR PREPHARYNGEAL REGION

FIG. 33.—DDAA. Anterior and right sides of graft failed to unite with host. 56 days. Graft larger than host head and controlled more posterior regions of host. Cephalic lobes, head shape, and epithelia of implant resembled those of host.

FIG. 34.—DDAP. Union complete. 21 days. New tissue partially covering anterior portion of implant. No appreciable change was observed during next 9 weeks.

FIGS. 35 (DORSAL VIEW) AND 36 (LATERAL VIEW).—DVAA. Union by posterior portion of dorsal surface only. 21 days. Short postcephalic outgrowth present. No appreciable change noted during next 9 weeks.

FIG. 37.—DVAA. Union complete. 21 days. Graft nearly inclosed by new tissue. Teratophthalmic outgrowth with dorsal epithelium of host and ventral epithelium of implant. No change noted during next 9 weeks.

Complete takes (*61 cases*).—Complete takes of cephalic grafts in the pharyngeal region generally induced tubular outgrowths which projected either from the dorsal or ventral surface of the host. (In 1 case no outgrowth developed, but in this it was noted from the first that the implant did not show the motor activity that was characteristic of all cephalic grafts.) When the eyes of the graft were subterminally located in the outgrowth, supernumerary photoreceptors developed at about the same level as those of the graft. This occurred even when the dorsal epithelium of the outgrowth was invaginated, as was the case when the outgrowth extended ventrally (Fig. 38). When the outgrowth developed at right angles to the anteroposterior axis of the graft, modification of the polarity of the graft was demonstrated by the development of eyes at right angles to those of the implant and occasionally the complete resorption of the original eyes (Fig. 39). When the eyes of the implant were located terminally, they did not regress, and none were induced in the host (Fig. 40). These facts may be interpreted as follows. When there is no part of the outgrowth representing a more anterior level than that of the eye-level, the eyes of the graft take up a terminal position in the outgrowth. In this case the rest of the graft and the induced portions of the outgrowth which correspond to more posterior levels do not produce eyes. On the other hand, if the eyes were subterminal, the more anterior portion of the outgrowth produces, at a certain distance from the tip, the physiological level at which eyes can develop or persist. Since the outgrowth is tubular, the dominance extends radially, resulting in a ring of eyes which in these experiments varied from two to eight, depending upon the diameter of the outgrowth and the "scale of organization" (Child, 1931).

Figure 41 shows the only complete take in this region in which a pharynx developed anterior to the graft. This was located in reversed polarity and may be assumed to have been induced by the implant. It was considerably smaller than the one which appeared posterior to the graft. That there had been a complete reversal of polarity of this portion of the host was proved when there was no regeneration following the removal of the host head a short distance in front of the anterior pharynx.

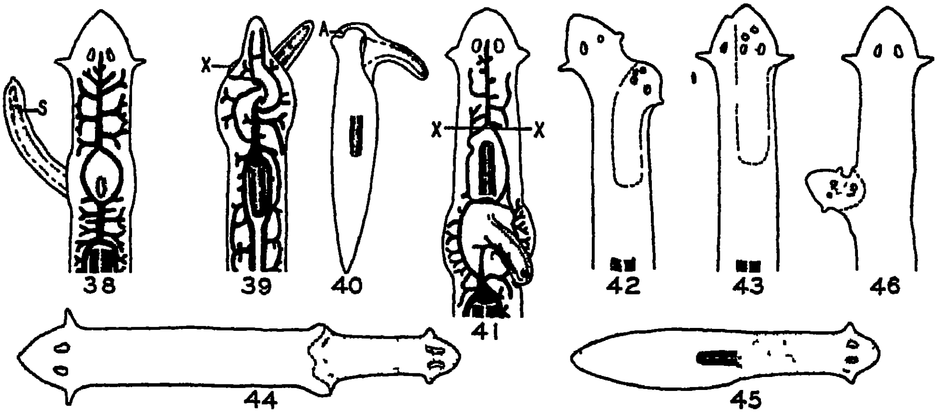
A gradual migration cephalad of grafts completely united with their hosts was noted in 9 cases. This was not as rapid as was observed in some cases of incomplete union, but in 1 case resulted eventually in fusion between the graft and host heads (Figs. 42 and 43). A rather significant feature of this migration was the disappearance of the cephalic lobe and eye of the host on the side nearest the graft. As the latter approached, these structures decreased in size and lost their pigmentation in a manner resembling stages in starvation. On the other hand, unpigmented new tissue was being laid down posterior to the graft, leaving a well-marked migration path behind. This indicated that host tissue was being used up anterior to the implant, while growth was taking place in the cells directly behind the graft. Consideration of these phenomena is reserved for the discussion.

In 8 cases grafts which united completely with the host were given a free surface. Four of these were given a free lateral and 4, a free posterior surface. In every case the anteroposterior axis of the resulting outgrowth was determined by the free surface and not by the original polarity of the graft. Figures 44 and 45 show a case in which a free posterior surface resulted in a 180° modification of graft polarity and the development of two additional eyes. These later fused with the original graft photoreceptors. Figure 46 shows a case in which a free lateral surface resulted in a modification of about 80° in the graft polarity.

In 3 cases the polarity was modified twice in the course of the observations. These were complete takes which were subsequently given a free lateral surface by a V-shaped cut in the side of the host. Figures 47 and 48 show the most clear-cut case. Freeing the lateral surface of the graft resulted in the development of a cephalic lobe at the anterior and another at the posterior margin of the graft and a photoreceptor beside the right eye of the graft (Fig. 47). These fused to form the right eye of the composite head. Meanwhile, as the graft migrated cephalad, the left side of the outgrowth fused with the right side of the host head. The right eye of the host and the two approximated cephalic lobes were resorbed (Fig. 48). Thus the polarity of the graft, after having been modified about 50°, finally, came to approximate its original condition.

Incomplete takes (52 cases).—The results of incomplete union differed from those of perfect takes in degree rather than in kind. In general the induced outgrowth of host tissue was greater and there was evidence of graft dominance extending a greater distance cephalad than in the case of complete takes. Of the four surfaces of the graft, the posterior united with the host most frequently (44 cases, 85 per cent).

GRAFTS IN THE PHARYNGEAL REGION



FIGS. 38, 42, 43, 44, AND 45 —*E. tigrina* into *E. dorotocephala*. FIGS 39 AND 46.—*E tigrina novangliae* into *E dorotocephala*. FIGS. 40 AND 41.—*E dorotocephala* into *E. tigrina novangliae*. Note that in all cases the reconstituted pharynx is posterior to original pharynx level (level of implantation).

FIG. 38.—DDAA. Union complete. 42 days. Tubular outgrowth developed covered with ventral epithelium and containing two supernumerary eyes (*S*, only one shown).

FIG. 39.—DDAP. Union complete. 35 days. Original eyes disappeared, and four pigmented areas developed. Tail-like outgrowth developed from right side of host anterior to implant. Host, decapitated at *X* (about 2 mm. from implant) 1 week earlier, completely failed to regenerate.

FIG. 40.—DDAP. Union complete. 42 days. Large graft (1.4 × 1.1 mm.). Ventral view. Large tubular outgrowth has developed with terminally located eyes. Decapitated 4 weeks earlier about 3 mm. from graft. Regeneration limited to small triangle of tissue (*A*).

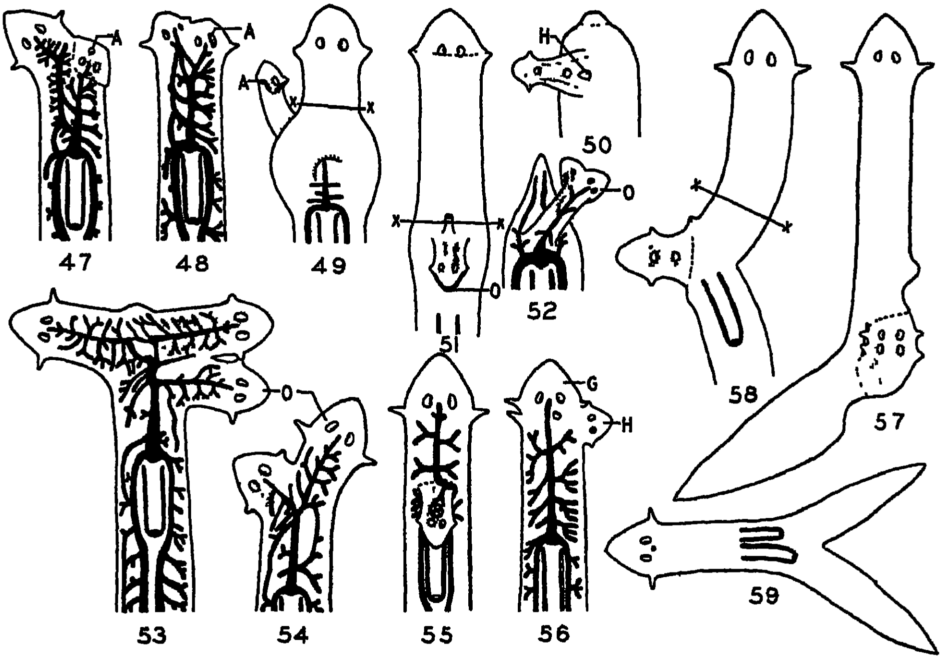
FIG. 41.—DDAP. Union complete. 28 days. Large graft (1.3 × 1.0 mm.). Pharynx with reversed polarity induced anterior to graft. Extent of graft dominance cephalad indicated by anterior reorganization of digestive tract. Host failed to regenerate after section at *X–X* 14 days later.

FIGS. 42 AND 43.—DDAA. Implanted to right of middle of host. Union complete. FIG. 42.—63 days. Path of graft migration indicated by new tissue. Nearest cephalic lobe and eye of host reduced in size with pigmented area entirely absent. Unpigmented outgrowth anterior to left graft eye. FIG. 43.—77 days. Entire right side of host head replaced by graft and surrounding tissues.

FIGS. 44 AND 45.—DDAA. Large graft (1.3 × 0.9 mm.). Union complete, but graft given a free posterior surface 7 days after implantation. FIG. 44.—31 days. Graft polarity modified 180°, and two pigmented areas developing behind original ones. Line between graft and host still distinct. FIG. 45.—74 days. Graft retained specific characters and reversed the polarity of a portion of host, as indicated by ciliary movement, orientation of pharynx, and regeneration of a tail after fission.

FIG. 46.—DDAA. Union nearly complete. 28 days. Left side of graft cut free from host 18 days after implantation. Left graft eye became right eye of outgrowth, and right graft eye eventually disappeared. Six weeks later graft had migrated cephalad, producing a Y-shaped compound resembling Figs. 54 and 61.

In 13 cases the graft united by three of its four cut surfaces, and tubular outgrowths open at both ends resulted. In 4 of the foregoing the free surface was lateral. In 3 of



FIGS. 47, 48, 50–59.—*E. tigrina novangliae* into *E. dorotocephala*. FIG. 49.—*E. dorotocephala* into *E. tigrina novangliae*. FIGS. 49, 50, 57–59.—DDAA. FIGS. 51–56.—DDAP.

FIGS. 47 AND 48.—42 and 63 days, respectively. Union complete. FIG. 47.—V-shaped incision made in right side of host opposite graft 1 week earlier. Eye (*A*) has developed distal to left graft eye. Cephalic lobes developing anterior and posterior to implant. Modification of graft polarity about 50°. FIG. 48 — Graft and host heads fusing. Right eye and auricle of host regressing. Unpigmented area of supernumerary eye (*A*) fused with right graft eye. With resorption of right side of host head, fusion was complete and polarity of graft returned to approximately original condition.

FIG. 49.—Right anterior corner of graft failed to unite with host. 28 days. Tubular outgrowth covered with ventral epithelium. Eye (*A*) developed opposite left graft eye. Graft polarity modified about 40°. Host decapitated at *x–x* regenerated normal head in 2 weeks.

FIG. 50.—Part of left and right sides of graft failed to unite with host. 21 days. Small head with two pigment spots has developed from left side of graft. Eye beside hole (*H*) on right side did not regress during 7 weeks of observation. Regeneration of host inhibited.

FIGS. 51 AND 52.—14 and 35 days, respectively. Anterior surface of graft failed to unite with host. FIG. 51.—Small pharynx with reversed polarity induced anterior to graft. Head has developed from host portion of tubular outgrowth (*O*). Sectioned at *x–x*, tail with two-branched digestive tract developed (Fig. 52).

FIGS. 53 AND 54.—35 and 96 days, respectively. Anterior and part of lateral surfaces of graft failed to unite with host. Large graft (1.5 × 1.1 mm.). FIG. 53.—Head (*O*) regenerated from free host surface behind graft. Right side of host tore apart at level of implant. FIG. 54.—Host head removed 3 weeks earlier failed to regenerate. During following 8 weeks host epithelia completely replaced those of graft.

FIGS. 55 AND 56.—35 and 105 days, respectively. Right anterior edge of graft failed to unite with host. FIG. 55.—Graft polarity modified about 70°, and third eye has developed. FIG. 56.—Graft epithelia replaced, but implant (*G*) has migrated cephalad. Greatly reduced host head (*H*) disintegrated 1 week later.

FIG. 57.—Sides of graft failed to unite with host. 31 days. Portion of host lateral to implant tore apart. New tissue anterior to graft has developed eyes and auricles with reversed polarity.

FIGS. 58 AND 59.—21 and 49 days, respectively. Left side of graft failed to unite with host. FIG. 58.—Host lateral to graft tore apart 1 week earlier. Modification of graft polarity indicated by position of auricles and of two pigmented areas. Following decapitation at *x–x*, tail and second pharynx developed in host anterior to graft. FIG. 59.—Graft eyes reduced to pigmented areas only.

these cases a modification in polarity was indicated by the position of the regenerated eyes and cephalic lobes. In the fourth, in addition to a large part of the left side of the graft failing to unite, there was a small hole between the graft and host on the right (Fig. 50). The right graft eye close to the hole did not regress, as generally occurred when there was a modification of 90° in the graft polarity (compare with Figs. 46, 58, and 59).

The 5 cases in which the anterior surface of the graft was free developed larger outgrowths than did the 3 cases mentioned above. In 3 of the 5 cases the outgrowths were ventral (Fig. 49); in 2, dorsal. In 2 cases there was a slight modification of polarity, and in 3 the free surface was approximately parallel with the eyes and polarity remained unchanged.

In 4 cases with reversed anteroposterior axis, in addition to the anterior surface of the graft, a small portion of each lateral surface failed to unite. In each case two cephalic lobes were regenerated and a tubular postcephalic outgrowth was induced. From the free surface at the posterior edge of the host window a secondary head developed. In 1 case (Figs. 51 and 52) a very small but functional pharynx developed in reverse orientation just anterior to the transplant. Following decapitation, a tail with the characteristic two-branched digestive tract developed from the anterior cut surface. In the second case the graft and secondary head tore through the right side of the host and thus obtained access to the substrate. During the next 3 weeks they were almost constantly pulling against the host head, taking on the appearance of the letter *T* (Figs. 53 and 54). Great difficulty was experienced in inducing this specimen to feed. During 4 weeks of starvation subsequent to the condition shown in Figure 53, the original host head was reduced to a size about equal to that of the graft head. In an attempt to prevent the excessive stimulation of three heads in the presence of food, the original head of the host was removed at this time. The cut surface healed, and the two remaining heads took up an anterior position. However, it was not until 3 weeks later that, as a result of being placed in the dark at feeding time (cf. above, p. 218), the specimen began taking food again. At this time it was only 1.6 mm. long, and the secondary host head was larger than that of the graft (Fig. 54). As it increased in size, the graft head grew more rapidly, until it again became equivalent to that of the host. During the 7 weeks of complete starvation the line of demarcation between graft and host remained quite distinct. However, as the specimen increased in size, the graft epidermis was observed to fade into host tissue along the line of separation until eventually the photoreceptors, head shape, and cephalic lobes resembled those of the host. This situation will be treated in the discussion.

In the third case, only a small portion of the right side of the graft was free, while a considerable portion of the left did not unite with the host. During regeneration of the graft a third eye appeared anterior to the left eye, and cephalic lobes developed opposite it and the right eye of the graft (Figs. 55 and 56). The implant increased in size as it migrated cephalad, and eventually dominated the host, while the host head became merely an appendage (Fig. 56). The fourth resembled the third except that more of the right side of the graft was free and there was no alteration of graft polarity. As a result of failure of the digestive tract of the graft to unite with a main branch of that of the host, the graft and the secondary host head received insufficient food and eventually regressed.

Two of the implants united by their anterior and posterior surfaces, leaving the larger portion of the lateral surfaces free. In both cases the eyes developed in the portion of the host in contact with the anterior part of the graft, i.e., in reversed orientation. In one, two structures resembling cephalic lobes developed in host tissue lateral to the induced eyes (Fig. 57). These appear to be another expression of the same situation observed in the development of photoreceptors in the tubular outgrowths resulting from complete takes. It is evident that the distance from the anterior edge of the graft to the graft eyes in one direction and to the induced eyes in the other was approximately equal. If the two lateral surfaces of the graft and host were fused, a situation resembling that in Figure 77 would result.

There was only 1 case in which a graft united with the host by one of the lateral surfaces. Two weeks after implantation it tore through the tissue on the left side of the host. The next week two pigment spots appeared distal to the left one of the graft, and a pharynx developed posterior to the level of implantation (Fig. 58). At this time the host head was removed. Instead of a head, a tail developed from the cut surface, and later a second pharynx appeared in reversed polarity (Fig. 59).

In 30 cases only the posterior surface of the graft united with the host. Figures 60 and 61 represent the typical result in this group. By an increase in size of the graft and a decrease in size of the host prepharyngeal region a Y-shaped compound resulted.

Five hosts tore apart either anterior or posterior to the implant. When the graft remained attached to the posterior piece, there was no regeneration of the host (Fig. 63). In these the graft formed the anterior portion of the worm. When the graft was attached to the anterior half, one or two tails developed from the exposed surfaces of the host. However, no pharynges developed, and the specimens gradually decreased in size.

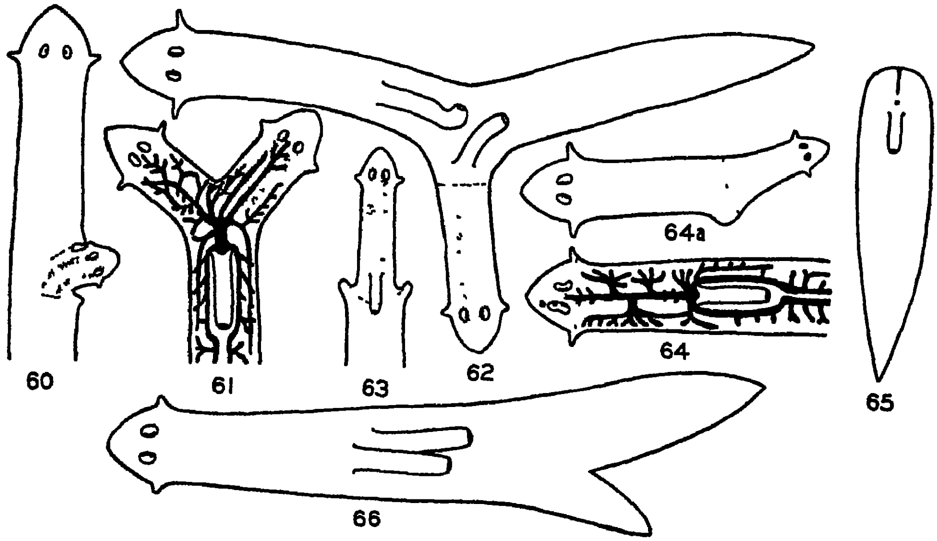
In 5 cases the windows were made by removing the dorsal wall and the underlying mesodermal tissues without cutting through the ventral wall. Grafts placed in these united with the dorsal surface of the host only. In addition there were 3 cases in which, although the usual procedure was followed, the ventral surface was not involved in the union. All 8 examples regenerated the triangular head shape and cephalic lobes but within 2 or 3 weeks became greatly reduced in size and showed evidences of lack of nourishment. When fed blood, it could be seen that no connection had been established between the digestive systems of graft and host.

Grafts placed in reversed dorsoventral orientation usually united with the host by their posterior surfaces, with the anterior tip of the graft bending back upon itself. When union of digestive tracts of graft and host occurred, the grafts persisted; otherwise they decreased in size and were lost.

Removal of the host head.—In 45 cases the host was decapitated 2 weeks or more after transplantation. Thirty were cut close to the graft (i.e., about 1–2 mm. anterior to it). In 18 of these regeneration of another host head was completely prevented (Figs. 39, 40, 50, 52). In the other 12 cases a normal head regenerated, but in 5 of these the grafts were regressing at the time of decapitation and so might perhaps be excluded from consideration. In 7 cases the section was made about 3 or 4 mm. from the graft. Five of these regenerated normal heads, and in only 2 cases was host regeneration inhibited (Fig. 59). In 8 cases the host was decapitated a short distance posterior to the host head. The only specimen that did not regenerate a normal head was one mentioned earlier (Fig. 41) in which the graft had induced a pharynx in reversed polarity anterior to the level of implantation. All the controls regenerated normal heads. It is evident that dominance of cephalic grafts does not usually extend much more than 2 mm. cephalad in large planarians such as were used in these experiments.

In 3 cases removal of the host body anterior to the graft, by section as near as possible to the graft, resulted in development of bipolars with a small but normal head instead of a tail at the posterior surface of the piece removed (Figs. 64, 64*a*). In these the polarity reversal was so firmly established that removal of the dominant region under which it had developed did not destroy it.

GRAFTS IN THE PHARYNGEAL REGION



Figs. 60–64.—*E. tigrina novangliae* into *E. dorotocephala.* DDAA. Fig. 65.—*E. dorotocephala,* control. Fig. 66.—*E. tigrina novangliae,* control.

Figs. 60 and 61.—Anterior surface of implant failed to unite with host and right side of host tore apart at level of implantation. Fig. 60.—14 days. Graft has regenerated cephalic lobes. Fig. 61.—42 days. Graft has increased in relative size and induced a pharynx. During next 9 weeks the two heads remained approximately equivalent.

Fig. 62.—Graft united to host by posterior surface. 35 days. The only case in which a pharynx with normal polarity appeared anterior to level of implantation.

Fig. 63.—Graft united by posterior surface. 14 days. Host tore apart anterior to implant 1 week earlier. No regeneration from exposed surfaces of host.

Figs. 64 and 64*a*.—Union complete. 35 days. 21 days after subsequent section of host just cephalad to implantation level. Fig. 64*a*.—Anterior part of host has regenerated a head on posterior surface. Fig. 64.—Posterior part of host containing implant has developed cephalic lobes. Two supernumerary eyes have appeared anterior to left graft eye.

Fig. 65.—Control. 7 days. Lateral margins of window united and animal tore apart. Posterior portion of animal developed single pigment spot and small pharynx but refused food.

Fig. 66.—Control. 63 days. Window in host remained open for more than a week. Incision made in right side 4 weeks after operation. Three weeks later tail-like projection appeared. Second pharynx appeared 2 weeks later.

Controls.—With only a few exceptions the tissue which was removed was replaced in the controls and a functional pharynx regenerated. In 1 case the window did not fill in completely, and a small head regenerated from its posterior edge. In another the two sides fused together, and, when the specimen tore apart subsequently, there was no regeneration at the anterior surface. A single median eye and a pharynx developed, but the piece would not feed (Fig. 65). In several controls in which there was little tissue along the sides of the window, the worms tore apart at this point. Later a head regenerated on the posterior piece and a tail on the anterior. In 3 cases after healing, tail-like outgrowths developed behind the level of operation. Figure 66 shows a case in which a pharynx developed in association with such an outgrowth.

GRAFTS IN THE TAIL (174 CASES)

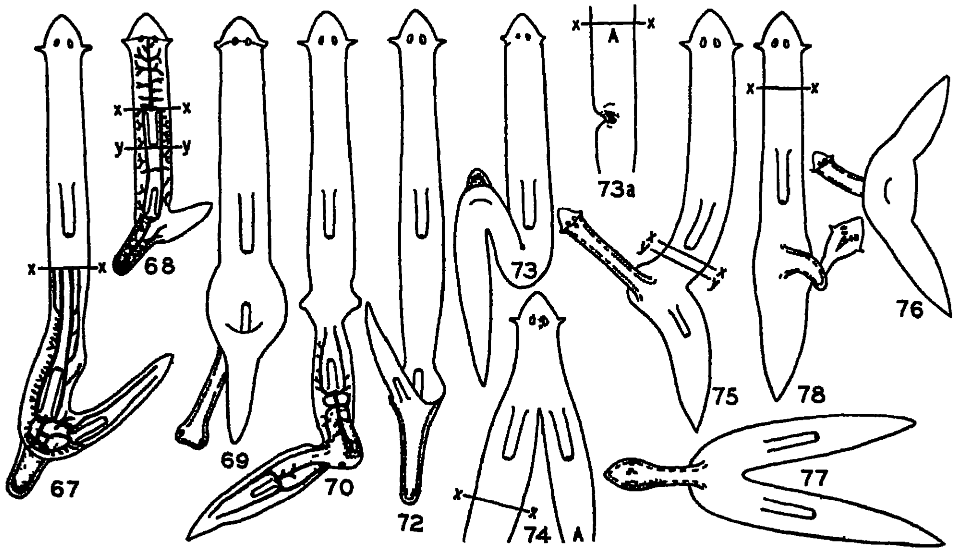
The tail proved to be the most labile of the regions studied. Cephalic grafts placed in this region induced the greatest outgrowths and produced the greatest reorganization of the host. Pharynges were induced equally well anterior or posterior to the level of implantation. They developed about the same distance anterior or posterior to the graft, but their lengths and diameters were proportional to the amount of tissue in which they developed. There were a few cases in which, in addition to a pharynx, a tail was induced anterior to the graft. Data on the range of dominance anteriorly showed that, unless the host was unusually long, the inhibitory power of the graft extended about to the fission zone of the host and occasionally into the pharyngeal level itself.

When the two-tailed forms produced by removal of the host heads were followed for long periods (6 months to a year), they were observed to fuse along the adjoining lateral margins, eventually producing double monsters. The fission products of these forms usually developed three or four eyes, two pharynges, and a more or less completely doubled digestive tract.

A few triangular implants containing chiefly ganglionic material were placed in the tail. Although containing less tissue than the rectangular grafts used generally in this study, they showed greater inductive capacities than the latter. No tendency to migrate was observed in implants in this level.

Complete takes (71 cases).—Cephalic grafts in the tail which united completely with their hosts produced the same type of tubular outgrowths as did complete takes in the pharynx. When the photoreceptors came to lie in the terminal portion of the outgrowth (43 cases), there was no alteration of graft polarity (Figs. 67, 68, and 69). When subterminal, supernumerary eyes developed in the induced tissue (Fig. 70). These outgrowths generally were larger than those resulting from grafts in the pharynx level (Pl. I, Fig. 71). Also, it usually was possible to observe that control by the graft extended some distance anterior to the level of implantation.

Pharynges were induced in the host in 19 cases. Of these, 13 developed two supernumerary pharynges, one posterior to the graft in normal orientation and the other anterior to it with reversed anteroposterior axis (Figs. 67, 70, and 72). In 4 of the 6 cases in which there was but one induced pharynx, it was posterior to the graft, while in the other 2 it was anterior (Fig. 69). In the latter the absence of a posterior pharynx may have been the result either of the small amount of host tissue posterior to the graft or of the proximity of the posterior tip of the tail, or both. In this connection it should be mentioned that there appeared to be a relationship between the length and diameter of the pharynx and the amount of tissue in which it developed, but the level at which it appeared was determined by the implant. Figures 67, 70, and 72 illustrate this situation. In these, both induced pharynges developed at approximately the same distance from the graft; but the posterior one, extending into the narrow tip of the tail, was both



FIGS 67, 68, 70, 72, 75, 76, AND 78.—*E. tigrina novangliae* into *E. dorotocephala*. FIG. 69.—*E. dorotocephala* into *E. tigrina novangliae*. FIGS. 73 AND 74.—*E. tigrina* into *E. dorotocephala*.

FIGS. 67 AND 68.—DDAP. Union complete. 21 days. Host fissioned anterior to implant and normal head and pharynx reconstituted.

FIG. 67.—Digestive tract reorganized and pharynx developed anterior, as well as posterior, to implant. Note that reversed pharynx is larger (also in Figs. 70 and 72). Following section at *x–x*, tail regenerated on posterior piece.

FIG. 68.—Digestive tract reorganized, and pharynx developed anterior to implant. Digestive tract was not seen in tip of tail, since no food entered. Normal head regenerated following decapitation at *x–x*. Tail developed following section at *y–y* 3 weeks later.

FIG. 69.—DDAA. Union complete. Graft has induced tubular outgrowth and pharynx with reversed polarity.

FIG. 70.—DDAP. Union complete. 14 days. Digestive tract reorganized and pharynges developed anterior and posterior to implant. Two eyes (only one figured) in new tissue posterior to graft.

FIG. 72.—Implants placed in both head and tail. DDAA. Union complete. 35 days. Graft in head has been incorporated into host. Pharynges have developed anterior and posterior to ventrally projecting tubular outgrowth induced by second graft.

FIGS. 73 AND 74.—Union complete. DDAA. 14 and 35 days, respectively. FIG. 73.—Ventrally projecting tubular outgrowth has developed and posterior part of host is under graft control. FIG. 73*a*.—Portion of animal after V-shaped incision was made, giving graft a free surface. One week later host fissioned at *x–x*. FIG. 74.—Original eyes reduced to pigmented spots. New eyes, cephalic lobes, and two pharynges have developed. Tail, instead of head, has regenerated at *A*. One week later animal fissioned at *x–x*.

FIG. 75.—DDAA. Left and posterior edges of graft failed to unite with host. 28 days. Graft polarity modified about 120°, and new eye has developed. Digestive tract (not shown) has reorganized. Tubular outgrowth and pharynx have developed posterior to implant. No regeneration occurred when sectioned at *x–x* 4 weeks later. After second removal (*y–y*), 8 weeks later, tail regenerated and second pharynx with reversed polarity developed anterior to implant.

FIG. 76.—DDAA. Anterior surface of implant failed to unite with host. 21 days. Tail, instead of head, regenerated on host following fission 1 week after implantation. Graft has induced a tubular outgrowth lined with dorsal epithelium.

FIG. 77.—DDAA. Very small part of right surface of graft failed to unite with host. 35 days. Implant induced tubular outgrowth with two eyes (one not shown) opposite those of graft and two pharynges. From hole two auricle-like structures developed. Anophthalmic head developed after fission of host 5 weeks earlier. When removed after 2 weeks, a taillike structure regenerated.

FIG. 78.—Triangular graft DDAA. Right side of graft and very small part of left failed to unite with host, leaving a hole on left. 56 days. Tubular outgrowth developed posterior to graft. Second pair of eyes developed between hole and lateral surface of outgrowth. Host regenerated normal head after section at *x–x*.

shorter and smaller than the anterior. Some of Santos' figures (Figs. 26, 29, 36, and 37) show the same condition, but he does not comment on the situation.

In 5 cases the host tissue on one side of the graft was removed, thus giving the graft a free surface. All 5 regenerated cephalic lobes and one or two eyes; and in each case the final polarity of the outgrowth was determined by the free surface (Figs. 73, 73*a*, and 74). There was no indication in these of migration such as was described for grafts in the pharynx.

Incomplete takes (103 cases).—Grafts in the tail which did not unite completely with their hosts regenerated their missing parts more or less completely, depending upon the amount of free surface. When union was nearly complete, tubular outgrowths developed; but when one or more sides of the graft remained free, the resulting outgrowth was flat. As was true for other levels, the polarity of the outgrowth was determined by the free surface. This was shown by the cases in which it did not coincide with the original anterior surface of the graft (Fig. 75).

In 16 cases union was nearly complete, and a tubular outgrowth developed. In 14 of these the outgrowth extended dorsally with the dorsal surface of the graft and host outside (Fig. 75). In the other 2 cases it was ventral with the dorsal surface inside (Fig. 76).

In 5 cases a very small portion of one edge of the graft did not unite, resulting in a hole between the graft and host. One or two cephalic lobes regenerated from this; and in the cases in which union was otherwise complete, a pair of eyes developed in the host portion of the outgrowth at the same level as those of the implant (Fig. 77). In 3 instances in which the graft united by only one surface, a second pair of photoreceptors developed between the hole and the lateral surface of the outgrowth, resulting in a compound head with three cephalic lobes and four photoreceptors (Figs. 78 and 79). These cases resemble rather closely that shown in Figure 57, in which a pair of eyes and two cephalic lobes developed in the new tissue anterior to an implant in the pharyngeal region. In 1 case a graft eye close to such a hole persisted for 14 weeks in a distinctly ectopic position, whereas usually photoreceptors at such a distance from the normal locus disappeared completely in 4–6 weeks (Fig. 82).

When the grafts in this level united by one edge, it was commonly the posterior (36 cases). In these there was the usual regeneration of missing parts by the graft head and reorganization of the host both anterior and posterior to the level of implantation. Since cases similar to those obtained in this study have been described and figured by Santos (1931), they will not be taken up in any detail. In 4 cases a lateral surface of the graft united with the host. In these, as with some of the foregoing, in which a portion of a lateral, as well as the posterior, surface was involved in the union, there was the usual modification in the polarity of the implant.

In a number of cases there were indications of the development of a tail between the host pharynx and the graft-induced one. These appeared first as slight swellings, which, as they grew, became supplied with a two-branched digestive tract. There were varying degrees of development of these outgrowths from slight projections to well developed tails. Figure 83 (Pl. I) is a photograph of one in which the supernumerary tail was well developed.

As was the case in other levels, reversal of the dorsoventral axis resulted in a proliferation of tissue at the line of union between graft and host. There was a tendency for the union to be superficial, involving only the two dorsal surfaces (Pl. I, Fig. 84). This was probably a result of the greater contraction of the ventral than the dorsal surface of the grafts when they were inverted. Incomplete unions were very common in these grafts, and the host window often tore apart directly anterior or posterior to the graft. In Figure 85 (Pl. I) we see a case in which the two portions of host tissue lateral to the graft developed into tails after the original host tail had torn off. However, when both dorsal and ventral surfaces united, there often was regeneration of one or two heads and tails. In one of these the dorsal surface of the host was united with the ventral surface of the graft, and in the other the ventral surface of the host with the dorsal surface of the graft. Figure 86 (Pl. I) shows an interesting example of this sort. In this case the host underwent fission directly behind the implant. A host head developed with reversed polarity, apparently induced by the graft, and with its ventral surface continuous with that of the graft. A single functional pharynx was induced, probably by the combined dominance of the two heads.

Cephalic grafts in the tail with the anteroposterior axis of the graft reversed retained their polarity in cases of complete union and reorganized the host equally well as did grafts in normal orientation. With reversed anteroposterior orientation, however, the graft formed the anterior, rather than the posterior, half of the outgrowth; and the induced eyes were posterior, rather than anterior, to the graft (Figs. 68 and 70).

In cases of incomplete union, cephalic grafts in the tail with anteroposterior axis reversed gave results indistinguishable from those whose anteroposterior axes coincided with those of the hosts.

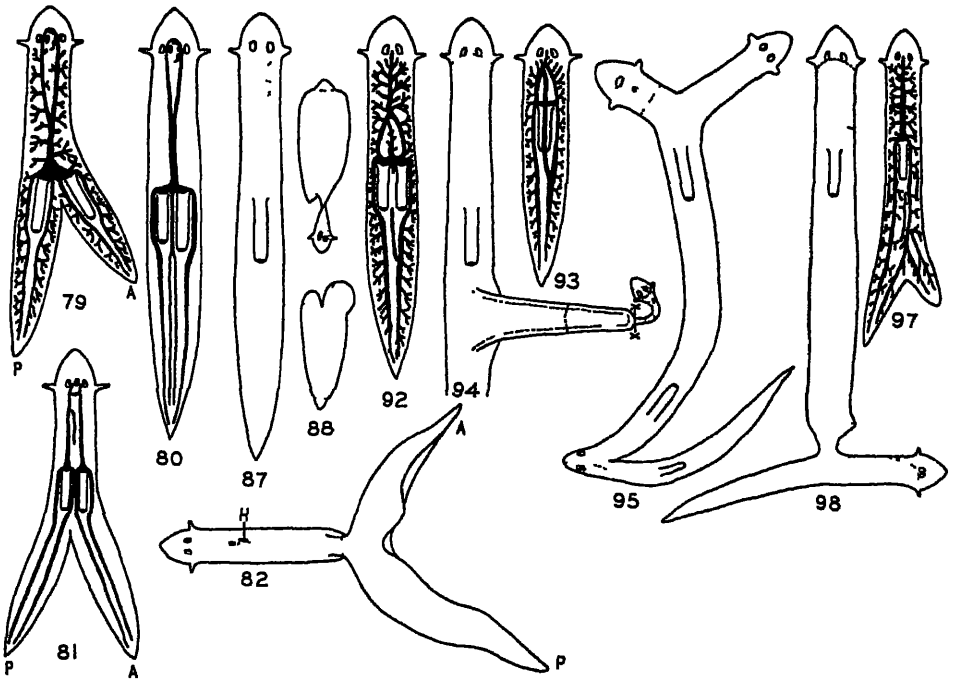
In 17 cases the host tore apart immediately anterior to the graft. In 14 of these the graft completely prevented regeneration from the exposed surfaces of the host. In the other 3 a tail, instead of a head, developed in the host. It was in grafts such as these that the persistence of the graft species characters was most marked. Figure 87 shows a case, 20 weeks after grafting, in which the species differences still were distinctly recognizable. The line between graft and host had faded out, but it was evident that the anterior and right portions of the compound were *E. tigrina* tissue while the posterior and left were *E. dorotocephala*. The persistence and replacement of graft tissues will be treated under "Discussion."

In 6 cases the posterior part of the host window tore apart, leaving the graft attached to the anterior piece of the worm. From the torn edges two tails developed in 1 case (Pl. I, Fig. 85), one tail in 3 cases (Pl. I, Figs. 89 and 90), and there was no regeneration in 2 cases (Fig. 88).

In 5 cases a pharynx was induced with reversed polarity. These grafts may be contrasted with similar ones in the pharyngeal region in which induction of a pharynx did not occur. In the latter the distance from the graft to host head was so short that there was no level of the gradient between them at which a pharynx could develop. However, in the case of implants in the tail the tissue between the two heads included the pharynx level, and reorganization by the graft expressed itself in the induction of a pharynx with reversed polarity.

In 40 cases the host head and part of the host body were removed 2 or more weeks subsequent to grafting. Of these, 10 were cut a short distance behind the host head, 10 about midway between the host head and the pharynx, 10 across the middle of the host pharynx, and 10 posterior to the pharynx. All of those cut close to the host head, and those cut in the prepharyngeal region regenerated normal heads anteriorly. Of those cut through the middle of the pharyngeal region, 4 regenerated normal heads and 6 developed tails, while all of those cut between the host pharynx and the graft regenerated

GRAFTS IN THE TAIL REGION



FIGS. 79–81, 92, AND 93.—*E. tigrina* into *E. dorotocephala.* FIGS. 82, 87, 88, AND 94.—*E. tigrina novangliae* into *E. dorotocephala.* FIG. 95.—*E. dorotocephala* into *E. tigrina novangliae.* FIGS. 79–82, 87, 92–95.—DDAA. FIG. 88.—DVAA.

FIGS. 79–81.—133 and 216 days, respectively. Left and a part of right side of graft failed to unite with host. FIG. 79.—Short outgrowth of tissue with two auricle-like structures developed from hole on right of graft. Tail, instead of head, regenerated from anterior surface (*A*) following fission. Fusion between two tails (*A* and *P*) beginning. FIG. 80.—Only main branches of digestive tract are figured. Lateral fusion of tails has resulted in doubled postpharyngeal region. FIG. 81.—Fission piece of Fig. 80. Note presence of three eyes and completely doubled digestive tract.

FIG. 82.—Triangular graft. 35 days. Right and very small part of left side of graft failed to unite with host. Left graft eye, close to hole (*H*), remained 14 weeks before final disappearance. Replacement of graft epithelia almost complete. Tail has regenerated from anterior end of host (*A*) following fission.

FIG. 87.—Union by posterior surface only. Host tore apart at implantation level 1 week after operation. 119 days. Graft has retained its specific characters, although line of union is no longer distinct.

FIG. 88.—Triangular graft. Posterior surface of graft failed to unite with host. 14 days. Graft polarity modified about 140°. Host fissioned anterior to implant and tore apart posterior to it. Graft inhibited anterior regeneration and dominated behavior of piece but was lost 2 weeks later.

FIGS. 92 AND 93.—Anterior surface of graft failed to unite with host. 182 days. FIG. 92.—Regenerated posterior third of fission piece with doubled digestive tract. Note two pharynges and partially doubled digestive tract. FIG. 93.—Piece fissioned from Fig. 92. Note single pharynx.

FIG. 94.—Triangular graft. Union by posterior surface of implant to dorsal surface only of host. 21 days. Original eye reduced to pigment spot, and two new ones have developed. Note long tubular outgrowth from host and small size of graft. Graft gradually decreased in size and eventually pinched off at *X–X* 6 weeks later. Induced outgrowth remained until termination of experiment on 105th day.

FIG. 95.—Double graft. Anterior implant in pharyngeal region, posterior in tail. Anterior united by right side only, posterior united completely. 21 days. Note three regions of influence in host: anterior under control of host head, middle under control of first graft, and tail dominated by second. Two weeks later, left eye of first graft was double, indicating 90° modification of polarity.

FIG. 97.—Control. *E. dorotocephala.* 42 days. Fission occurred at level of window; and, following, regeneration of normal head, outgrowth containing two-branched digestive tract appeared.

FIG. 98.—*E. dorotocephala.* Control for Fig. 73. 21 days. Right side of host window tore apart, and teratophthalmic head regenerated.

tails. In addition to this experiment, there were 58 cases of fission posterior to the pharynx but anterior to the implant. Of these, 33 developed tails anteriorly, 20 showed no outgrowth of tissue at all, and 17 regenerated heads. Some of the fissions here recorded occurred during the first week after operation. Of these, 16 developed normal heads, leaving only 1 case in which a normal head developed after the reorganization effects had begun to appear.

In all cases in which tails were induced, there was a tendency for the two tails to become parallel and fuse (Pl. I, Fig. 91). In those which were observed for long periods of time (up to 15 months) the two tails gradually approximated, until finally a worm with doubled pharyngeal and postpharyngeal regions resulted. When fission of this double animal occurred, the fission pieces usually developed two to four photoreceptors and a more or less completely doubled digestive tract, consisting, in the most extreme cases, of two anterior branches, two pharynges, and four posterior branches (Figs. 80 and 81). In 1 instance sixteen successive fission pieces from a single graft showed this condition. Sonneborn (1930) produced doubled forms of *Stenostomum* as a result of treatment with lead acetate. These abnormalities were passed from one generation to another by fission, and therefore he considers them to be hereditary modifications. From the same point of view, the modifications described above could be considered hereditary, since they depended upon a self-perpetuating pattern persisting from parent to offspring. However, it appears to the writer that a most important feature of this "inheritance" has not been taken into account. This is the fact that all the "offspring" resulting from fission are, in reality, parts of the parent and must necessarily partake of such axial modifications of the parent as the doubling described by Sonneborn. In the cases reported here, it is extremely improbable that there was any modification of the hereditary units which might persist through sexual reproduction. An experiment was performed with the planarians which, because of the very small number of animals, was inconclusive except in one respect. Two large double specimens of *E. dorotocephala* were cut transversely into three approximately equal pieces and allowed to regenerate. The two anterior pieces regenerated a tail and a single pharynx. The two middle pieces, each containing two pharynges, regenerated a normal head and a tail containing a three-branched digestive tract. The posterior pieces regenerated normal heads and partially doubled digestive tracts, containing two pharynges and three posterior branches (Fig. 92). During the second week of regeneration fission occurred in the posterior pieces, and the fission pieces both reconstituted a single pharynx and almost normal posterior intestinal branches (Fig. 93). Further experiments along this line are necessary before the factors involved can be determined. However, since the same animal could produce single as well as double individuals, the persistence of the latter condition could hardly be called hereditary, even in the sense of Sonneborn.

Triangular grafts (33 cases).—The first grafts which were made in this study were triangular grafts in the tail region, made according to the method of Santos (1931). These implants contained less tissue than the rectangular grafts which were used later, but contained more of the cephalic ganglia. The postcephalic outgrowths induced by these were generally larger than those of the rectangular grafts, but often the host digestive tract did not unite with those of the grafts, and the latter did not persist (Fig. 94).

In only 3 of the 33 cases of triangular grafts did the ventral surface of the graft unite with that of the host; in the other 30 cases it united with the dorsal surface of the host and the host ventral surface healed over. As a result of this, tubular outgrowths which ended blindly were induced even in cases of incomplete union (Figs. 78 and 94), and the frequency of union between graft and host digestive tract was very much less than in rectangular implants. Among the rectangular grafts there were only 3 cases in which the ventral surface of the host did not unite with some part of the graft.

Double grafts (18 cases).—The effect of the level in which a graft is placed was strikingly illustrated in a series of 18 double grafts. Figure 72 shows the results of simultaneous implantation of cephalic grafts in the head and in the tail of the host Both were complete takes; but the graft in the head healed without leaving any traces, while that in the tail induced a tubular postcephalic outgrowth and two pharynges.

Figure 95 illustrates the result of simultaneous transplantation in the pharynx and tail. In this case the anterior graft united by the right side only, while the posterior united by all surfaces. Three well-marked regions of influence can be seen. That controlled by the host head extended about to the level of implantation of the first graft. The first graft controlled the movements of the host a short distance anterior to it and posteriorly about half the distance to the pharynx induced by the second graft. The second graft controlled about equal distances anteriorly and posteriorly. Figure 96 (Pl. I) is a photograph of a case in which grafts placed in the pharynx and tail both united with the host by all four surfaces. The three regions of influence evident in Figure 95 can also be seen in this illustration.

Controls.—The large majority of the controls replaced the parts removed, and within 2–4 weeks were normal again. The exceptions consisted chiefly of cases in which the lateral edges of the window united, effectively preventing food from reaching the tip of the tail, which eventually either tore apart or disintegrated. In two cases a tail-like outgrowth developed behind the level of operation, and one of these was observed to be supplied by two branches of the digestive tract (Fig. 97). In one case one side of the window tore apart, and from it a teratophthalmic head regenerated. This dominated the behavior of the more posterior part of the specimen and completely reorganized it (Fig. 98).

DISCUSSION

TRIANGULAR VERSUS RECTANGULAR GRAFTS

The increased frequency of takes by using rectangular, instead of triangular, grafts was mentioned in the section on "Methods." In addition to this, the use of rectangular grafts has clarified the problem of polarity of the implants. In cases where implant and host united completely, Santos (1931) shows figures of small monophthalmic outgrowths of undetermined polarity and states that they were the usual result. In the experiments here, such outgrowths were common in triangular implants but were not observed in rectangular grafts. The small outgrowth of tissue in the triangular type grafts is thought to be the result of union of the grafts with the dorsal surface only of the hosts, since it was also observed in cases of incomplete union in which the host ventral surface was not involved. The monophthalmic condition was found by the writer to be the result of the partial removal of one of the graft eyes during operation and the absence of any regeneration. With rectangular grafts the ability of complete takes to reorganize host tissues was in many cases equal to that of incomplete takes. Pharynges were induced posterior to the implant in grafts in the pharynx; and in the tail, induction by complete takes extended both anteriorly and posteriorly (Figs. 39, 40, 41, 67, 68, 70, 72, and 96). In fact, 1 of the

2 cases in which a pharynx was induced anterior to a graft in the pharynx level was a case of complete union (Fig. 41). This is in contrast to the findings of Santos (1931) with triangular grafts.

POLARITY

The anteroposterior polarity of cephalic grafts is shown in these experiments to be extremely labile. In cases of complete union there was little evidence that the axes of the grafts were modified except in anteroposterior grafts in the head. In these it was not possible to determine positively whether the implant retained its original axial orientation or not, but it seems likely that reversal occurred. There was no indication of lack of co-ordination of movement between host and implant or of cilia beating in opposite directions. The direction of the ciliary beat has been used by Rustia (1925) and by F. S. Miller (1937) as a criterion of the polarity of planarians and seems to be reliable.

Grafts containing any appreciable amount of free surface always showed corresponding modifications of polarity. These modifications ranged from very slight changes, in which a single photoreceptor developed in close proximity to one of the graft eyes, through cases of 90° modification with two photoreceptors developing at right angles to the original ones (Figs. 46, 47, 48, 55, 56, 58, and 59), to complete reversal in cases in which the posterior surface of the graft remained free (Figs. 30 and 31). The latter type of modification was rare because the posterior surface of the grant united more commonly than any other, but the results were consistent.

The lability of the axial organization of the most complex and highly differentiated region of a planarian is quite remarkable. Under certain conditions eyes appear, and under others they regress and disappear. On the other hand, polarity varies with the position of the free surface or surfaces. These conditions are very difficult to reconcile with a concept of polarity such as that of Harrison (1921, 1936), involving models used in stereochemistry. In order to do so, it would be necessary to develop additional theories to explain the reorientation of the hypothetical molecules with each modification in polarity. In a case such as is shown in Figures 47 and 48, three such modifications must be accounted for. In addition, the correlation between the exposed free surface and the polarity of a cephalic graft is unaccounted for by such a theory. Similar difficulties are encountered in attempting to interpret the observed modifications of polarity in these experiments with the space-lattice concept of Przibram (1921). On the other hand, the theories of Goetsch (1929) seem to be, in reality, chiefly a restatement of observed conditions rather than any constructive hypothesis which may aid in a true understanding of the significance of this general feature of organisms.

By contrast with the foregoing, the gradient concept which has a large body of experimental proof requires no additional hypothesis to account for the determination of polarity by a free surface. According to this concept, polarity is a function of the presence of a dominant region and its morphogenetic activities along the anteroposterior axis. One of the most characteristic features of the dominant region is its relatively high rate of physiological activity, and so the problem of the origin of polarity resolves itself into the origin of a region of great activity. That regenerative processes are characterized by increased physiological activity of the cells at the free surface has been demonstrated in a large number of forms. That the polarity of a planarian regenerate is independent of the nervous system of the piece is indicated in the histological studies by Flexner (1898) on *P. torva* (syn. *E. tigrina novangliae*) and by Keiller (1910) on *Planaria* *simplicissima* (syn. *Curtisia foremanii*), which showed that the ganglia of the regenerate develop independently of the old nervous system. Similarly, Beyer and Child (1930) concluded from experiments on the reconstitution of lateral pieces of *E. dorotocephala* that the nervous system is not an essential factor in determining the polarity of the regenerate.

Accordingly, we may suggest that the changes in polarity of the grafts resulted from activation of the cells of the free surface, which set up a new dominant region in the regenerate, and that this region gradually expanded its physiological and morphogenetic control until the entire graft-controlled tissue was repolarized. There has been little work throwing light on gradient conditions within the head, but it is possible that the slope here is very slight. The great lability of the polarity of cephalic grafts would seem to suggest this.

As was also found by L. V. Morgan (1906) and Santos (1931), the dorsoventral organization of the planarian pieces used in these experiments was not susceptible to modification by any of the situations which arose as a result of grafting. In every case the dorsal and ventral epithelia of grafts retained their peculiarities, even though, as time passed, they gradually became replaced by host tissues.

The results of these grafting experiments emphasize the fact that the tail of planarians which undergo fission is physiologically quite different from the head. It has been recognized for some time (Hyman, 1916) that the posterior, growing region of certain forms involves special conditions within the primary anteroposterior gradient. That this region is definitely polarized is shown by the fact that tails are regenerated from posterior surfaces of pieces from this level and heads always appear at the anterior surfaces. However, the experiments reported here, and those of Santos (1931) and of F. S. Miller (1937), indicate that polarization is by no means as strong posterior to the pharynx in *E. dorotocephala* and *E. tigrina* as in more anterior levels, and emphasize the fact that the high physiological activity and great regenerative powers of this region should be more thoroughly investigated from a gradient point of view.

REPLACEMENT OF GRAFT BY HOST TISSUE

With few exceptions there was a gradual replacement of graft epithelium by that of the host. This has been described on page 219; and it was noted that the photoreceptors, as well as the epithelia, were replaced. Since the eyes are a part of the nervous system, this fact would suggest that the other tissues of the graft, as well as those which could be observed, probably were also involved in the replacement.

The replacement of tissues was especially rapid in small grafts which were composed largely of ganglionic tissue. On the other hand, large grafts which contained, in addition to approximately the same amount of nervous tissue, some prepharyngeal tissue retained their specific characters for a considerable longer period, and in some cases did not show any appreciable replacement of epidermal structures during the entire course of the observations. Even in these, however, the line between graft and host faded out in time and the tissues of that region took on an appearance which was intermediate between the two species.

The structure of the anterior part of a planarian seems to offer a suggestion as to the factors which determine whether a heteroplastic graft will or will not retain its specific differences. The head of a planarian consists largely of nervous and muscular elements with very little parenchymatous tissue. It is from the latter that the more specialized cells are normally differentiated. By contrast, in the anterior prepharyngeal region nervous tissue is limited chiefly to the nerve cords; and, although the diverticula of the digestive tract occupy a much larger space than in the head, there is considerably more packing or parenchyma tissue. Curtis and Schultze (1934) have found also that there are more "formative cells" in this than in the ganglionic region.

Grafts which retained their specific characters all united with the host by only a portion of the cut surfaces. In 2 cases they were grafts in reversed dorsoventral orientation (Fig. 29), and in the others they were grafts in normal orientation which united by only one surface. Thus, it will be noted that they were cases in which the grafts, because of free anterior and lateral surfaces, were able, to a considerable extent, to regenerate lost parts. With the regeneration of the parenchymatous tissue in the prepharyngeal region they were supplied with cells of their own species with which to replace those that became senescent, and thus they retained their specific characters. On the other hand, it is suggested that the situation, in the grafts which gradually developed host characters, was that the implanted tissues contained insufficient parenchyma cells to make the necessary replacements, and that this was accomplished by host cells.

Of considerable interest in this connection are the observations on page 230. It is well known (Stoppenbrink, 1905) that starvation causes a reduction first of the reproductive system and then of the parenchyma, digestive tract, and muscles, leaving the nervous system practically unaffected over a long period. In the case shown in Figures 53 and 54 it seems likely that starvation had reached a point where the implant was almost, if not completely, devoid of parenchymatous tissue. When feeding was reinstituted, it seems likely that host parenchyma migrated into the implant, for it soon began to take on the characteristics of the host species, although during the entire period of starvation the specific characters of the two components could be recognized. That host tissues do invade the graft is indicated by Steinmann's findings (1933). Using hosts vitally stained with *Cresylechtviolett*, he observed that the digestive tract of the host invaded the graft for a considerable distance.

GRAFT MIGRATION

In the experimental section was noted the migration of cephalic grafts in the pharyngeal region. When this migration is compared with regeneration of the controls, some striking similarities are seen. When a window is cut in a planarian, the surrounding tissues contract and a more posterior level is brought into contact with a more anterior one. During regeneration new tissue forms between the two levels until they are again separated by approximately the same distance as before. Thus, in the process of regeneration the more posterior level may be said to have "migrated" back to its original level. In terms of gradients, we may say that, when two gradient levels come in contact, new tissue forms between them until the lower level is again separated from the higher by cells representing the intervening levels of physiological activity.

It might appear, at first sight, that graft migration is fundamentally the same situation excepting that the difference in physiological level between the tissues in contact is greater. New tissue would be expected to form between graft and host until the intervening levels of the axial gradient are restored. This explanation does not, however, fit all the facts, for, according to it, the reconstituting pharynx would be expected to appear at the same level as the original one, that is, in the new tissue. This occurred in the controls, but in the experimentals in every case but one the reconstituted pharynx was to be found caudad in the old host tissue.

The indications are that the experimental procedure in some way rejuvenates the piece which is grafted. If this is the case, it would explain the hypertrophy of the grafts and graft induced tissues in comparison to the uninfluenced regions of the host. It would also interpret the observation that on one occasion, when the laboratory temperature rose suddenly over a week-end, the grafts of a number of specimens disintegrated while the hosts remained intact. The greater muscular activity of grafts would also tend to indicate a higher general physiological activity, such as is characteristic of young planarians. If this should prove to be the case, then the facts observed regarding graft migration could be interpreted on the basis of a physiologically younger axis developing at the expense of the host tissues. Two processes are involved in a case like Figures 42 and 43: first, the development of a morphogenetic field behind the graft, and second, the elimination of tissue in front of the graft. That host tissues were actually destroyed is shown by the disappearance of structures on the left side of the host head as the graft approached, while the left side of the graft gave little evidence of suffering from its proximity to another dominant region.

If the graft is placed in anteroanterior orientation, it would be expected to migrate toward its level of origin; while if in anteroposterior orientation, it would migrate away from it. This is in agreement with observations of Santos (1931) on postcephalic grafts and with those reported here.

INDUCTION AND RANGE OF DOMINANCE

The very slight inductive ability of cephalic grafts in the head is in striking contrast to grafts in the pharyngeal and tail regions. Implants in the head induced outgrowths only when there was an exposed cut surface; and the supernumerary eyes which appeared when the grafts were ectopic or in reversed anteroposterior orientation are, at best, very questionable cases of induction. Since in two of the controls supernumerary eyes developed, there is a possibility that those of the experimentals were the result of regeneration by the host under slightly abnormal conditions. The occasional auricle-like structures may also have been regenerated rather than induced.

In the pharyngeal level, cephalic grafts have greater inductive power than in the head, though less than in the tail. Short to medium postcephalic outgrowths developed, and the level at which reconstituting pharynges appeared was considerably farther posterior than in controls. Since these developed by reorganization of tissue which had not been injured in the operations, and since no control showed such development, we may say that these pharynges were graft-induced.

Placed in the tail, cephalic grafts induced reorganization cephalad as well as caudad. Santos (1929, 1931) reported the induction of pharynges in reversed polarity by grafts in postcephalic regions but did not show the condition of the digestive tracts of these cases. Feeding the animals blood has made it possible, in many of the cases described here, to observe that the digestive tracts were completely reorganized between the graft and the induced pharynges both anterior and posterior to the implant. There were in certain cases structural evidences of induction at a still greater distance from the implant. In those a second tail developed between the original host pharynx and the more anterior graft-induced one (Fig. 83). Observations on the behavior of the compounds also indicated that graft dominance extended beyond the more anterior of the induced pharynges. During locomotion there were well-defined zones under the influence of the host and of the graft. Stimulation of the graft resulted in contraction of the host as far anterior as the induced tail. On the other hand, stimulation of the host, unless severe, had little or no visible effect upon regions posterior to the induced tail.

Inductive capacity is associated with the ability to inhibit independent differentiation by tissues in the neighborhood of the inducer. The distance along the axis that this controlling action extends has been called "range of dominance" (Child, 1924). With the dominant region anterior, the controlling action extends in a posterior direction only. However, when a dominant region is placed in the pharyngeal region or tail, it may extend its control in an anterior direction as well. It is possible to obtain a rough measure of the anterior range of dominance of a graft by decapitation of the host at various distances anterior to the implant. Data from the decapitation experiments showed that the range of graft dominance was greater in the tail than in the pharyngeal region. In the case of grafts in the pharyngeal region "inhibitory dominance" seldom extended more than 2 mm. cephalad. On the other hand, grafts in the tail usually inhibited head regeneration from a cut surface as far anterior as the host pharynx, a distance of about 4–6 mm.

ROLE OF THE NERVOUS SYSTEM IN PLANARIAN INDUCTION

The fact that the heads of planarians are largely composed of nervous tissue would suggest the possibility that this tissue was responsible in some manner for the resistance of the cephalic level to induction. If this were the case, there should be a marked reduction in host resistance when implants were made directly behind the head in the anterior prepharyngeal region. Figures 31, 32, 33, 34, 35, 36, and 37 show that this was not the case. The results of Okada and Sugino (1934) on the Japanese *P. gonocephala* are of some interest in this connection. Their reported inductions of pharynges by implants from prepharyngeal regions show that in the Japanese species ganglionic material is not necessary for the reorganization of lower levels in the gradient. Santos (1931) did not report any inductions by implants from this level in *E. dorotocephala* and *E. tigrina*; but this matter probably should be reinvestigated, since it is known that pharynges will reconstitute in prepharyngeal pieces even if regeneration of a head is completely inhibited (Child, 1911*c*).

SUMMARY

1. A technique of operation involving rectangular transplants and ice as anesthetic gave a high percentage of takes between *E. dorotocephala* and *E. tigrina*. Four hundred and twelve cases of successful implantation of cephalic grafts were studied. Of these, 105 were in the head, 113 in the pharyngeal level, and 174 in the tail. Twenty were placed in the anterior prepharyngeal region for comparative purposes.

2. Union between the digestive tract of the implant and host was necessary for persistence and growth but not for regeneration of the graft. In all levels a free surface was necessary for regeneration by the transplant and determined its anteroposterior axis. Modifications of polarity up to 180° were observed. When union between graft and host was complete, there was no graft regeneration, and polarity apparently remained unmodified. Dorsoventrality of the grafts in all cases remained unchanged during the entire period of observation.

3. In most cases there was a gradual centripetal replacement of graft epithelia by

those of the host. There was some evidence that tissues other than the epithelia were also involved. When the amount of union between graft and host was slight, this replacement did not take place.

4. Cephalic grafts in the head commonly united with the hosts by all surfaces and became incorporated in them. Their epithelia and photoreceptors eventually were replaced by host tissue.

5. When the anteroposterior axis of hosts and grafts in the head region did not coincide, whether by accident or as a result of implantation in anteroposterior orientation, supernumerary photoreceptors occurred.

6. When grafts in the head did not unite completely, very short postcephalic outgrowths developed; and often from the free edge of the host window a more or less complete secondary head appeared.

7. In grafts in dorsoventral orientation there was more or less formation of new tissue between graft and host. In some cases this resulted in the development of additional heads, one surface of which developed from the implant and the other from the host.

8. Grafts in the nonganglionic, anterior prepharyngeal region showed very little more ability to induce than did those in the head, indicating that resistance to induction is not dependent upon the amount of nervous tissue present.

9. Grafts in the pharyngeal region induced larger postcephalic outgrowths than did those in the head. These were flat in cases of incomplete union but tubular in complete or nearly complete takes. A pharynx was induced posterior to the level of implantation in almost every case. In two cases a pharynx reversed in polarity was induced anterior to the graft.

10. A gradual migration was noted in a number of grafts in the pharyngeal region. This was most evident during inanition and was characterized by the appearance of new tissue behind the graft and the disappearance of host tissue anterior to it.

11. Cephalic implants in the tail region induced the largest outgrowths of tissue and the greatest reorganization of the host. This included reorganization of the two branches of the postpharyngeal digestive tract into a single prepharyngeal branch, development of pharynges anterior and posterior to the implant, and in a few cases a supernumerary tail between the pharynx of the host and that induced anterior to the graft.

12. Triangular cephalic grafts containing more anterior levels but less actual tissue than the rectangular implants showed greater inductive capacities than the latter.

13. Decapitation of the host anterior to the implants showed that the range of dominance of grafts in the pharyngeal region usually did not extend more than about 2 mm. cephalad. In the tail region, graft dominance usually extended about to the posterior tip of the host pharynx.

14. Double forms were developed from the posterior fission pieces of grafts with two tails. These possessed two pharynges, doubled prepharyngeal branches of the digestive tract, and more or less doubled postpharyngeal branches. Perpetuation of these by fission occurred, but they certainly could not be called hereditary modifications.

15. The gradient in host resistance to induction was strikingly illustrated in cases in which grafts were simultaneously implanted in different levels of the same host.

16. In the discussion the following topics are treated: (*a*) triangular versus rectangular grafts, (*b*) polarity, (*c*) replacement of graft by host tissue, (*d*) graft migration, (*e*) induction and range of dominance, and (*f*) role of the nervous system in planarian induction.

LITERATURE CITED

ABELOOS, M. 1930. Recherches expérimentales sur la croissance et la régénération chez les planaires. Bull. biol. France et Belgique, 64:1–140.

BEYER, K. M., and CHILD, C. M. 1930. Reconstitution of lateral pieces of *Planaria dorotocephala* and *Planaria maculata*. Physiol. Zoöl., 3:342–65.

BROWNE, E. N. 1909. The production of new hydranths in *Hydra* by the insertion of small grafts. Jour. Exper. Zoöl., 7:1–24.

BURT, D. R. R. 1934. The capacity of different regions of *Pelmatohydra oligactis* Pall. to form head or foot. Jour. Exper. Zoöl., 68:59–94.

CHILD, C. M. 1911*a*. Experimental control of morphogenesis in *Planaria*. Biol. Bull., 20:309–31.

———. 1911*b*. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. I. The axial gradient in *Planaria dorotocephala* as a limiting factor in regulation. Jour. Exper. Zoöl., 10:265–320.

———. 1911*c*. Studies, etc. III. Physiological dominance of anterior over posterior regions in the regulation of *Planaria dorotocephala*. *Ibid.*, 11:187–220.

———. 1921. Studies, etc. XI. Physiological factors in the development of the planarian head. *Ibid.*, 33:409–33.

———. 1924. Physiological foundations of behavior. New York: Henry Holt & Co.

———. 1927. Experimental localization of new axes in *Corymorpha* without obliteration of the original polarity. Biol. Bull., 53:469–80.

———. 1929*a*. Physiological dominance and physiological isolation in development and reconstitution. Arch. f. Entw'mech., 117:21–66.

———. 1929*b*. Lateral grafts and incisions as organizers in the hydroid, *Corymorpha*. Physiol. Zoöl., 2: 342–74.

———. 1931. Experimental modification of the scale of organization in the reconstitution of *Tubularia*. *Ibid.*, 4:165–88.

———. 1932. Determination of new axes by lateral grafts and incisions in *Corymorpha* in relation to the gradient and dominance. *Ibid.*, 5:593–627.

———. 1935. Dominance of hydranths induced by grafts in *Corymorpha*. Jour. Exper. Zoöl., 71:375–87.

CURTIS, W. C., and SCHULTZE, L. M. 1934. Studies upon regeneration. I. The contrasting powers of regeneration in *Planaria* and *Procotyla*. Jour. Morphol., 55:477–513.

FLEXNER, S. 1898. The regeneration of the nervous system of *Planaria torva* and the anatomy of double-headed forms. Jour. Morphol., 14:337–46.

GEBHARDT, H. 1926. Untersuchungen über die Determination bei Planarienregeneraten. Arch. f. Entw'mech., 107:684–726.

GOETSCH, W. 1926. "Organisatoren" bei regenerativen Prozessen. Die Naturwiss., 41:1011–16.

———. 1929. Das Regenerationsmaterial und seine experimentelle Beeinflussung. (Versuch zur einheitlichen Beurteilung der regenerativen Erscheinungen.) Arch. f. Entw'mech., 117:211–311.

HARRISON, R. G. 1921. On relations of symmetry in transplanted limbs. Jour. Exper. Zoöl., 32:1–136.

———. 1936. Relations of symmetry in the developing embryo. Collecting Net, 11:217–26.

HUXLEY, J. S. 1935. The field concept in biology. Trans. on Dynamics of Development, 10:269–93.

HUXLEY, J. S., and DE BEER, G. R. 1934. The elements of experimental embryology. Cambridge University Press.

HYMAN, L. H. 1916. An analysis of the process of regeneration in certain microdrilous oligochaetes. Jour. Exper. Zoöl., 20:99–163.

———. 1925. The reproductive system and others characters of *Planaria dorotocephala* Woodworth. Trans. Amer. Micr. Soc., 44:51–89.

JANICHEN, E. 1897. Beiträge zur Kenntnis des Turbellarienauges. Zeitschr. f. wissen. Zool., 62:250–88.

KEILLER, V. H. 1910. A histological study of regeneration in short head pieces of *Planaria simplicissima*. Arch. f. Entw'mech., 31:131–44.

KENK, R. 1935. Studies on Virginia triclads. Jour. Elisha Mitchell Sci. Soc., 51:79–125.

LANG, P. 1913. Experimentelle und histologische Studien an Turbellarien. I. Mitteilung. Heteromorphose und Polarität bei Planarien. Arch. f. mikr. Anat., 82:256–70.

MILLER, F. S. 1937. Some effects of strychnine on reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl., 10:276–98.

MILLER, J. A. 1937. Studies on heteroplastic transplantation in triclads. I. Cephalic grafts between *Euplanaria dorotocephala* and *E. tigrina*. Doctor's dissertation, University of Chicago Library.

MORETTI, G. 1911. Sulla trasposizione delle varie parti del corpo nella *Planaria torva* (Muller). Arch. ital. anat. embr. (Firenze), 10:437–60.

MORGAN, L. V. 1906. Regeneration of grafted pieces of Planarians. Jour. Exper. Zoöl., 3:269–94.

MORGAN, T. H. 1903. The control of heteromorphosis in *Planaria maculata*. Arch. f. Entw'mech., 17: 683–95.

MURRAY, M. R. 1931. In vitro studies of planarian parenchyma. Arch. f. exper. Zellforsch., 11:656–68.

MUTZ, E. 1930. Transplantationsversuche an *Hydra* mit besonderer Berücksichtigung der Induktion, Regionalität und Polarität. Arch. f. Entw'mech., 121:210–71.

OKADA, YÔ K., and SUGINO, H. 1934. Transplantation experiments in *Planaria gonocephala*. I. Proc. Imp. Acad., 10:37–40.

PRZIBRAM, H. 1921. Die Bruch-Dreifachbildungen im Tierreiche. Arch. f. Entw'mech., 48:205–444.

RAND, H. W.; BOVARD, J. F.; and MINNICH, D. E. 1926. Localization of formative agencies in *Hydra*. Proc. Nat. Acad. Sci., 12:565–70.

RAND, H. W., and BROWNE, A. 1926. Inhibition of regeneration in planarians by grafting: technique of grafting. Proc. Nat. Acad. Sci., 12:575–81.

RUSTIA, C. P. 1925. The control of biaxial development in the reconstitution of pieces of *Planaria*. Jour. Exper. Zoöl., 42:111–42.

SANTOS, F. V. 1929. Studies on transplantation in *Planaria*. Biol. Bull., 27:188–98.

———. 1931. Studies on transplantation in *Planaria*. Physiol. Zoöl., 4:111–64.

SONNEBORN, T. M. 1930. Genetic studies on *Stenostomum incaudatum*. II. The effects of lead acetate on the hereditary condition. Jour. Exper. Zoöl., 57:409–39.

STEINMANN, P. 1933. Transplantationsversuche mit vital gefärbten Tricladen. Rev. suisse de zool., 40:269–72.

STOPPENBRINK, F. 1905. Der Einfluss herabgesetzter Ernährung auf den histologischen Bau der Süsswassertricladen. Zeitschr. f. wissen. Zool., 79:496–547.

STRELIN, G. S., and TRIFONOVA, A. N. 1935. Fiziologicheskii gradient. Arch. Russes Anat. Hist. et Embryol., 14:56–73.

WEISS, P. 1935. The so-called organizer and the problem of organization in amphibian development. Physiol. Rev., 15:639–74.

WILLIER, B. H.; L. H. HYMAN; and S. A. RIFENBURG. 1925. A histochemical study of intracellular digestion in triclad flatworms. Jour. Morphol. and Physiol., 40:299–340.

PLATE I

PHOTOGRAPHS OF LIVING ANIMALS

FIGS. 28–30, 33, 71, 83–86, 89–91, AND 96.—*E. dorotocephala* into *E. tigrina novangliae.* FIGS. 31 AND 32.—*E. tigrina novangliae* into *E. dorotocephala.*

FIG. 1.—*E. dorotocephala.*

FIG. 2.—*E tigrina.*

FIG. 3.—*E. tigrina novangliae* possessing mid-dorsal light stripe, type used in these experiments. Note pharynx (*P*).

GRAFTS IN THE HEAD REGION

FIG. 28.—DVAA. Union complete. 273 days. Graft epithelia replaced by host tissue. Dorsoventral axis unaltered and no regeneration of host eyes. Same specimen shown in Fig. 27, 1 week after operation.

FIG. 29.—DVAA. Union by posterior part of dorsal surface only. 280 days. Graft (*G*) has regenerated head, tail, and functional pharynx. Species specificities kept during 10 months of observation.

FIG. 30.—DVAP. Posterior surface of graft failed to unite with host. 21 days. No host head developed during 133 days of observation.

GRAFTS IN THE ANTERIOR PREPHARYNGEAL REGION

FIG. 31.—DDAA. Union complete. 175 days. Two auricle-like structures developed lateral to implanted eyes. Graft epithelia replaced by host tissues.

FIG. 32.—DDAA. Anterior surface of implant failed to unite with host. 180 days. Host tissue disintegrated to right of graft. Epithelia, head shape, and cephalic lobes of implant (*G*) typical of host rather than graft.

GRAFTS IN THE TAIL

FIG. 71.—DDAA. Union complete. 252 days. Note tubular, graft-induced outgrowth with pharynx (*P*) posterior to, and with pharynx (*P′*) and very small projection anterior to, implantation level.

FIG. 83.—DDAA. Union by graft posterior surface only. 133 days. Reorganization of digestive tract with induction of pharynx (*P*) anterior as well as posterior to implant. Note tail (*T*) between host pharynx and anterior graft-induced one.

FIG. 84.—DVAA. Union by dorsal surfaces only. 21 days. Host fissioned during first week. Outgrowth of tissue does not involve ventral surface of host. Intestinal diverticula of graft failed to unite with those of host. Implant subsequently regressed and finally disintegrated.

FIG. 85.—DVAP. Union by posterior and left edge of graft. 28 days. Host window tore apart at posterior angles 1 week after operation, and sides have developed into tails. Unpigmented areas of three graft eyes (one *R* regenerated) seen through ventral surface.

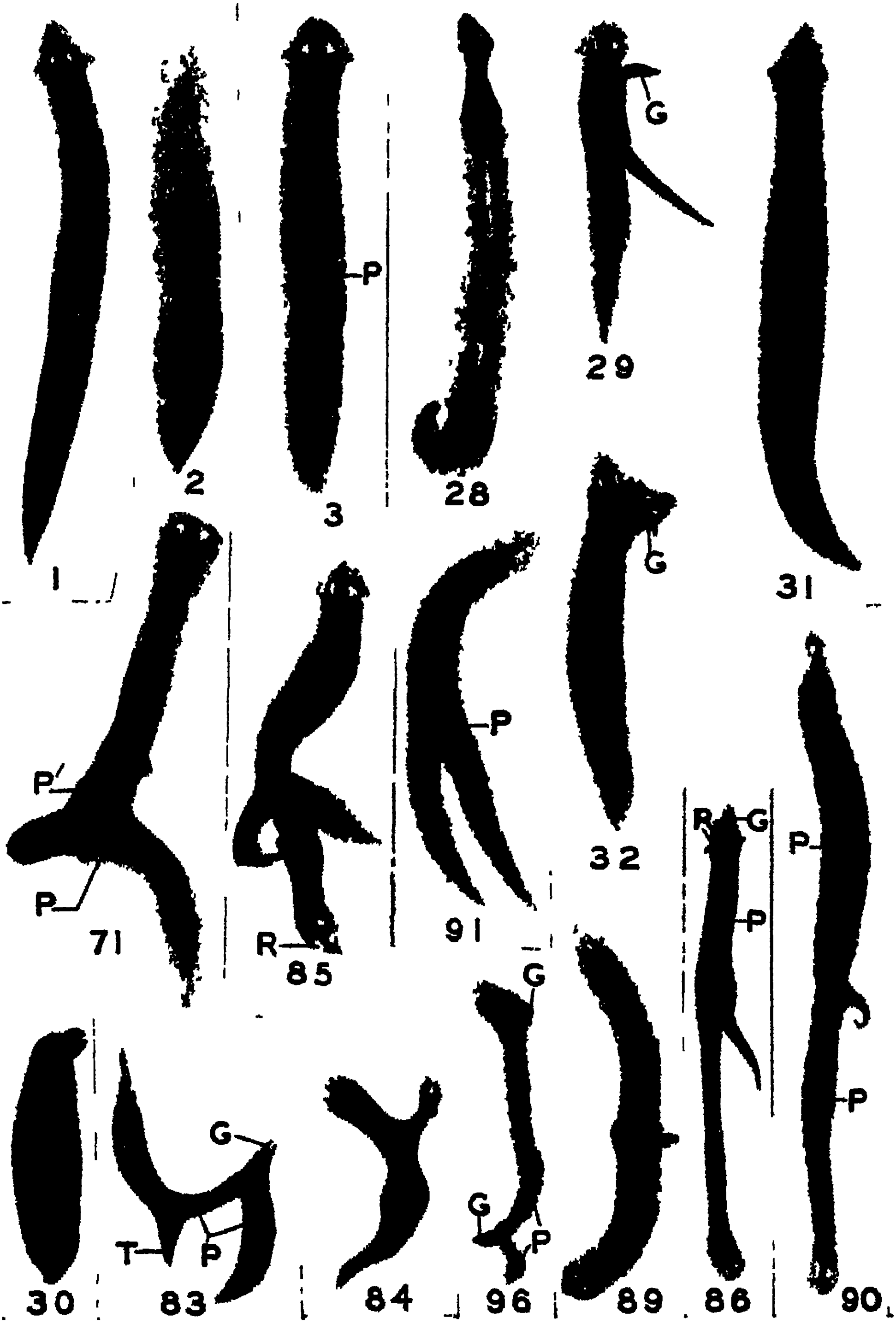
FIG. 86.—DVAP. Union complete. 70 days. Host fissioned immediately behind implant (*G*), and regenerated head (*R*) instead of tail opposite that of graft. Both share common ventral surfaces and pharynx (*P*) with reversed polarity.

FIGS. 89 AND 90.—DDAP. 56 and 70 days, respectively. Anterior surface of graft failed to unite with host. Window in host tore apart posterior to implant. FIG. 89.—Postcephalic outgrowth and pharynx with reversed polarity induced by graft. Small tail-like appendage developed from window. Graft epithelia replaced by host tissues. FIG. 90.—Photographed after feeding blood to show pharynges (*P*).

FIG. 91.—DDAP. Union by graft posterior surface. 154 days. Fission piece. Tail and pharynx (*P*) with reversed orientation developed in host anterior to implant. Fusion of tails along lateral surfaces in progress.

FIG. 96.—Two grafts (*G*). Both DDAA, union complete. 35 days. Teratophthalmic head regenerated on host after section 2 mm. anterior to first graft. Note extent of control of each head caudad and cephalad, and reorganization of digestive tract and induction of pharynx (*P*) anterior to second graft.

PLATE I



Physiological Zoölogy

Vol. XI JULY 1938 No. 3

MODIFICATIONS OF DEVELOPMENT IN RELATION TO DIFFERENTIAL SUSCEPTIBILITY OF THE BRUCHID (COLEOPTERA) EGG TO KCN DURING DIFFERENT METABOLIC PHASES

(Twenty-one figures)

ALFRED BRAUER
Department of Zoology, University of Kentucky

THERE is an abundance of experimental evidence showing that organismic pattern is physiological, rather than morphological, in its earliest manifestation. It has likewise been demonstrated in numerous instances that early physiological patterns are factors in the establishment of the permanent morphological pattern (Child, 1924). Bellamy (1919) has shown that a physiological pattern precedes the development of the morphological pattern in the frog's egg, and that the latter can be modified at will by altering the former.

In the insect the egg at the time of oviposition is not a mosaic of prospective values, as was formerly supposed. It has now been demonstrated for insects of several orders that such values are determined progressively soon after the laying of the egg (Seidel, 1929, 1934; Schnetter, 1934; Brauer and Taylor, 1936).

During this period of determination the pattern of the insect embryo is labile and may be modified by disturbances such as constrictions involving the anterior fourth of the presumptive germ band or cautery of the same area. After these experiments regulation forms, such as complete embryos posterior to the constriction, are common.

For the purpose of analyzing further the determination of pattern in the bruchid egg, especially as regards the determination of the primary axis, it was decided to attempt its experimental modification at various periods by altering physiological processes through their differential susceptibility to KCN. It has been demonstrated (Hyman, 1919) that cyanide depresses the rate of oxygen consumption, and its differential depression by this method is the subject of many papers (see Child, 1924, p. 76).

The specific questions to which answers are sought are: (1) Is there any evidence of physiological pattern in the egg during blastokinesis or during that period when morphological pattern is known to be labile? (2) After formation of the blastoderm, is there any indication of axial pattern before it becomes visibly apparent? (3) If physiological patterns are apparent in the egg, is it possible to produce characteristic modifications of development by their alteration?

It is a pleasure gratefully to extend acknowledgments to Dr. C. M. Child for his interest in the problem and for valuable suggestions which he offered.

Methods

Gravid female pea weevils were confined to vials containing sterilized peas for 15-minute intervals, after which the peas were replaced by fresh ones. The peas with the newly laid eggs attached were divided into from three to five lots of 6 to 10 eggs each, depending upon the number of eggs available at one time. After incubating the eggs to the desired age at a temperature of 30° C., the different lots were immersed in KCN M/100 for varying periods of time. The process was repeated until a sufficient number of eggs of each stage had been treated to give the experiment value. The minimum number of eggs for a time period of treatment at a particular developmental stage is 14, the maximum number 50, the average 25. Thus for example, several lots of eggs, each with from 6 to 10 eggs, which had been incubated for 2 hours, were treated for various periods of time. One lot was treated for 10 minutes, another for 15 minutes, another for 20 minutes and so on, until egg lots had been treated from 10 to 50 minutes or the lethal period of 100 per cent of the eggs at this stage.

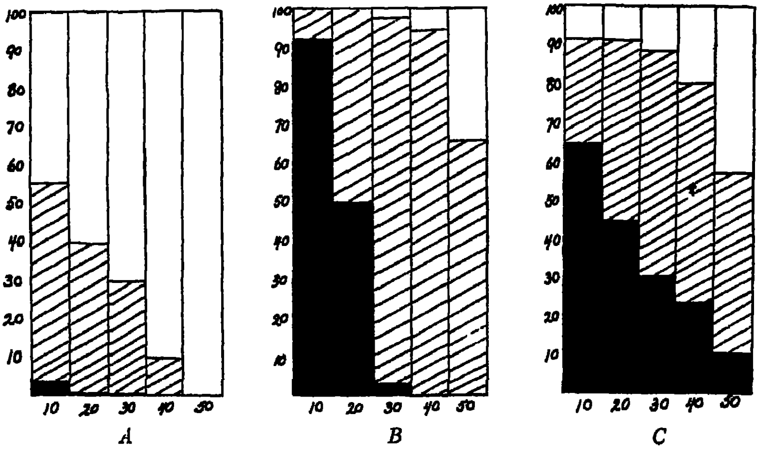


Fig. 1.—Graph showing percentage of complete development (solid bars), teratological development (shaded), and no development (unshaded). *A*, first phase. *B*, second phase. *C*, third phase. Abscissas, time of treatment in minutes. Ordinates, percentages.

After treatment in cyanide the eggs were thoroughly washed, dried on filter paper, and incubated. Those which were to be examined for immediate effects of treatment were returned to the incubator for only 1–2 hours, while those to be examined for teratological development were incubated for 48 hours longer. The eggs were then removed from the peas under dissecting binoculars, fixed, stained *in toto*, and cleared. From this point they were first studied entire, and in many cases drawings were made. They were imbedded by the celloidin-paraffin method. The celloidin blocks containing eggs were often filed away in vials of Kornhauser's clearing mixture. In this they could be re-examined at will, since the blocks were entirely transparent, or they could be imbedded in paraffin for sectioning.

SUSCEPTIBILITY TO KCN AND MODIFICATIONS OF DEVELOPMENT

I. FIRST PHASE: ½—6½ HOURS' INCUBATION

A. GENERAL SUSCEPTIBILITY

Eggs of less than 6½ hours' incubation show almost immediate susceptibility when immersed in the solution, to the extent that they give grossly anomalous development or no organized development after further incubation. After only 10 minutes' treatment, 45 per cent of the eggs give no development; 41 per cent give axiate but highly anomalous development; while only 4 per cent give complete development. The latter include complete embryos which show microcephaly, and other slight defects of general form. From this point unorganized development increases progressively up to 50 minutes' treatment, after which no eggs were found to give axiate development (Fig. 1*A*).

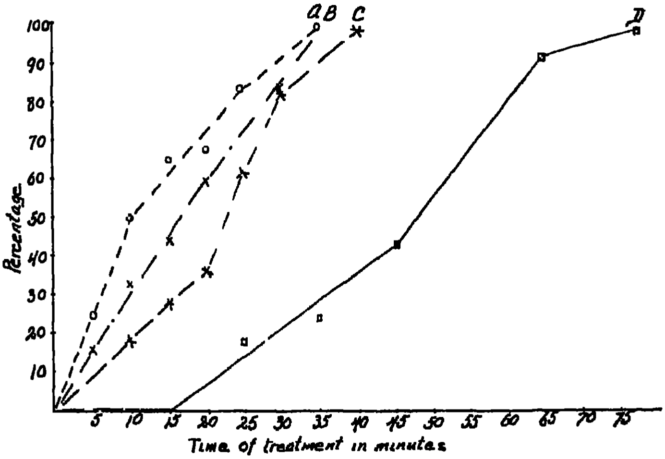


FIG. 2.—Graph showing comparative susceptibility in first two phases. Abscissas give time of treatment in minutes. Ordinates show percentage of eggs killed. *A*, *B*, *C*, treatment during first phase at 1, 2½, and 4½ hours, respectively. *D*, treatment at 7½ hours (second phase) for comparison.

There is a difference in the initial reaction of eggs to treatment during this interval of time. Eggs treated immediately after oviposition react to treatment more quickly than do eggs 2½ hours old, and the latter more quickly than eggs 4½ hours old. The time required for killing lies between 35 and 40 minutes for any age less than 6½ hours (Fig. 2). Eggs during the first phase, though showing no axial development, frequently give considerable tissue proliferation. It has been found that some eggs, seemingly undergoing no development upon gross examination, nevertheless show some cellular proliferation when sectioned. Any axial development may always be distinguished in stained and cleared eggs.

Developmentally, eggs range from 16 to 200 cleavage nuclei or more during this phase. There is considerable range of difference in any batch of eggs all the same age.

B. DIFFERENTIAL SUSCEPTIBILITY: GROSS MODIFICATIONS

A variety of developmental modifications are exhibited by treated eggs of the first 6½ hours, which, however, are found to fall under one of three types, as follows:

Unorganized terata: In some of these no development whatsoever is apparent in the stained and cleared eggs. Eggs are quite homogeneous throughout. These are uncommon when treated for short periods, and increase in frequency for treatments of longer duration. In greater number are the eggs which contain darkly stained clumps of material which are amorphous and scattered. They are found laterally, anteriorly, or posteriorly. The formless clumps have been identified as unorganized terata.

Axial terata: Of these, there are varied examples. The most anomalous appear much like the darkly stained clumps of the preceding group excepting that they are axiate and exhibit segmentation. The anterior portion is sometimes differentiated into cephalic lobes. Others are quite complete embryos but are dwarfed and distorted (Fig. 4).

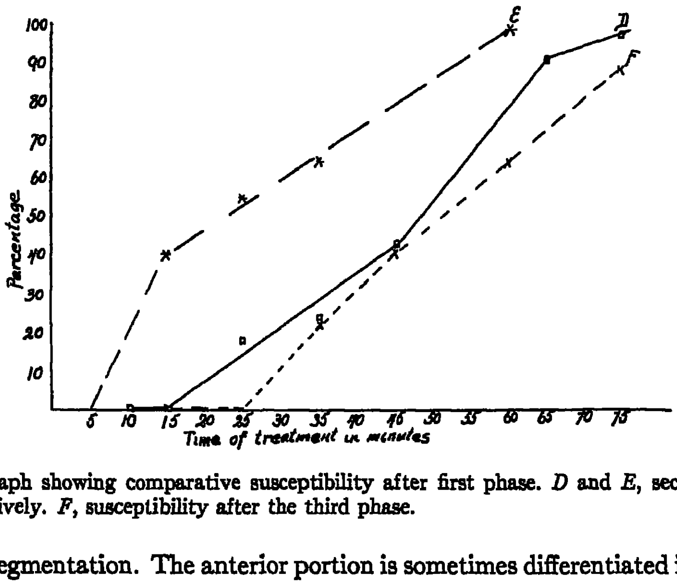


FIG. 3.—Graph showing comparative susceptibility after first phase. *D* and *E*, second and third phases, respectively. *F*, susceptibility after the third phase.

Complete embryos: These occupy the entire ventral surface of the egg and are differentiated into head, thoracic, and abdominal segments. The embryos are anomalous in that they are microcephalic giants with poor development of head and mouth parts. The bodies are very broad. Segmentation is not distinct, and the ventral chain of ganglia is poorly developed. Terata of this type appear in eggs less severely treated and having a more complete recovery than those of the preceding forms (Fig. 5).

1. *The egg immediately after treatment: cytology.*—For this study, eggs were treated after 2, 4, and 6 hours' incubation. After treatment they were returned to the incubator for only a short time (½–1 hour) or until it was thought that sufficient time had elapsed for any injury to become visibly apparent. They were treated for 25 minutes, or sufficiently long to inhibit development of all but a small percentage of the eggs. They were then removed from the peas, fixed, and sectioned for microscopic examination.

In all such eggs examined, cleavage nuclei were found undergoing mitotic division. No injury appears in the cells which have not reached the egg cortex. They retain their staining capacity and in all respects appear normal. Nuclei which have reached the cortex but have not entered it, at 4–6 hours, remain under the cortex and do not become engulfed by it in the characteristic reaction exhibited by cleavage nuclei and cortical cytoplasm of normal eggs. The nuclei, however, still appear normal and exhibit mitosis. Nuclei already in the cortical cytoplasm at the time of treatment soon lose their capacity to hold stain, and become shrunken.

The effects of treatment first become noticeable in the oöplasm, and particularly in the cortical cytoplasm. This appears contracted and shrunken, while the ooplasm just under the surface begins to undergo a vacuolation, apparently due to a contraction of the shrunken cytoplasm, which leaves the yolk globules behind in the interstices (see Fig. 13). It is the cortical cytoplasm of the treated eggs which appears to lose its capacity to react with cleavage nuclei arriving at the surface.

In eggs treated prior to 4 hours' incubation, little differential susceptibility is discernible. The cortical cytoplasm generally is very susceptible, and effects of treatment are easily seen in micro sections.

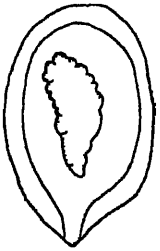


FIG 4

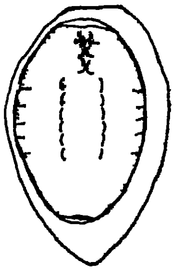


FIG 5

FIG. 4.—Axiate dwarfed embryo occupying a portion of ventral egg surface (camera lucida).

FIG. 5.—Microcephalic giant of first phase (camera lucida).

2. *The egg after further incubation.*—Unorganized development: In section, eggs of this type are found to contain darkly staining patches of cells lying near the surface in various parts of the egg. These tissue patches appear to be the result of a continued proliferation of cleavage cells after treatment of the egg. The original cleavage cells arriving at the egg cortex, especially in the ventral surface near the anterior border, fail to become incorporated into the severely injured cortical cytoplasm. They continue to proliferate, however, and instead of forming a blastoderm in the cortical cytoplasm, form a clump of cells under the surface. These clumps are syncytia in which cellular boundaries are not distinguishable (Fig. 6).

In these eggs the cortical cytoplasm, as well as much of the internal cytoplasm, shows irreparable injury. On the ventral surface, cortical cytoplasm is usually entirely absent. On the lateral and dorsal surfaces, sometimes short strips remain. The cellular masses always appear to occur where there is some cortical cytoplasm; but in eggs of unorganized development, no blastoderm is formed, however.

The vacuolated areas within the egg are probably due to destruction of internal cytoplasm and subsequent shrinking, causing the yolk globules to mass together in the interstices.

Eggs treated between 4 and 6½ hours' incubation already have a few cleavage cells imbedded in the cytoplasm of the anteroventral region. After treatment these cells may either fail to proliferate (and merely degenerate) or they may divide without further synthesis of protoplasm and form, over a portion of the surface, a thin, flattened layer containing nuclei. Whether these cells degenerate or whether they form a regulation blastoderm in these areas probably determines whether the subsequent development is unorganized or organized.

If any differences in reaction are apparent, the cortical cytoplasm of the ventral surface near the anterior end shows the first evidence of susceptibility, followed by that of the anterior end and by that of the more posterior ventral surface, while that of the dorsal side is last to show the effects (Fig 7)

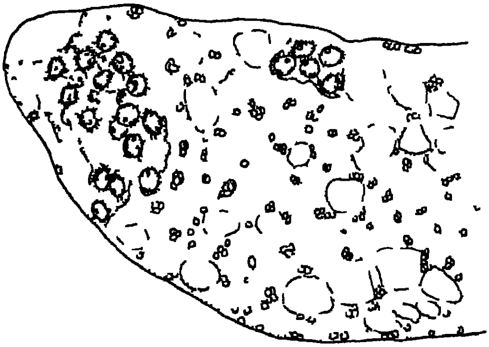


Fig 6

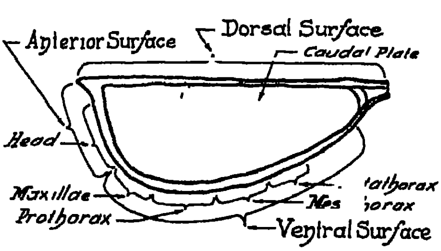


Fig 7

Fig 6—Section of first-phase egg with unorganized development Note that little cortical cytoplasm remains in egg

Fig 7—Diagram of a medial longitudinal section of an egg showing surfaces and the presumptive regions of the egg surface

Differential effects of cyanide treatment during the first phase become definitely distinguishable in the cortical cytoplasm of eggs treated after 4 hours' incubation, or during the latter part of the first phase. In these the first and most apparent effects are in the cortical cytoplasm of the ventromedial surface near the anterior border. Here the cortical cytoplasm becomes very thin, and under it vacuoles form in the ooplasm. The effects diminish gradually from this center elliptically in all directions. The dorsal cortical cytoplasm is the last to show injury effects. The egg region showing greatest susceptibility is the region at which cleavage nuclei normally first reach the cortical cytoplasm, and where a short time later the greatest concentration of cleavage cells occurs (Fig 8).

When an egg treated at 4½ hours and incubated 2½ hours longer is superimposed over one not so severely treated and which has undergone further development, the injured areas of the two show fairly close coincidence. The affected area in the developed embryo extends from the prothoracic region up through the region of the mouth parts (Fig. 9).

Organized axial terata: In this group are eggs containing embryos which range from small darkly staining dwarfed terata to large, more complete embryos with fairly well-formed segments. All of these embryos have cephalic lobes and some body segmentation.

The more poorly formed lie far laterally in the egg, usually in the angle between the convex ventral surface and the flattened dorsal surface. The head lobes are small, and the body segments are often asymmetrical. In others, head lobes are quite well developed. The body segments appear well formed but are highly anomalous.

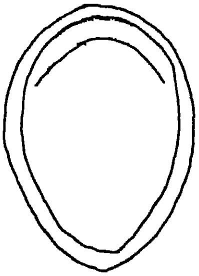


Fig. 8

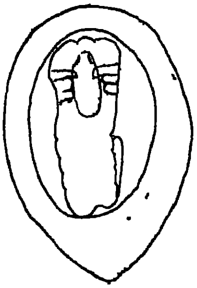


Fig. 9

Fig. 8.—Diagram of ventral view of an egg showing differential susceptibility to KCN. Densely stippled area shows greatest susceptibility.

Fig. 9.—Outline drawing of a first-phase egg with embryo, showing deficient area from prothoracic region through maxillary region (solid lines), superimposed over a first-phase egg (stippled outline) fixed 2 hours after treatment. Stippled egg shows region of ventral surface most affected by treatment (inner eliptical stippled lines). Outline by camera lucida.

Sections show the anterior end of these embryos to be histologically differentiated. The ectoderm of the head lobes shows severe injury in that the cells are very irregular in form and outline, with nuclei of varying size. Head segments contain an entodermal canal with the cells of this germ layer considerably better developed than the ectoderm.

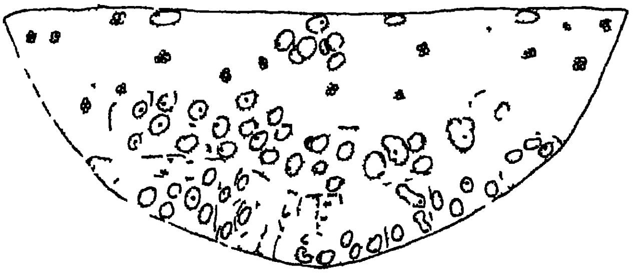


Fig. 10.—Section of organized embryo of first phase. Injury is most apparent in ectoderm. Development of mesoderm is abnormally great.

Posterior to the head region the cortical cytoplasm appears to have been injured considerably more severely. Here ectoderm, normally the most prominent layer, is present, though poorly formed. Its cells are irregular or flattened. There is much evidence of cellular disintegration and occasional evidence in some parts of mitotic activity on the part of nuclei. The mesodermal layer is thicker and usually more abundant than in the normal embryo (Fig. 10). Farther posteriorly, development of ectoderm is less complete and mesoderm becomes reduced, though there are differences in individual embryos.

The outstanding difference between the organized terata of this series and the unorganized formations of the former is that in all of the organized embryos an ectoderm is present. This undoubtedly means that the cortical cytoplasm of the egg was less severely injured than in the former series or that recovery took place, so that cleavage cells reacting with this cytoplasm formed ectoderm. The ectoderm of these embryos is undoubtedly responsible for the formation of the inner germ layers by the mitotic division of ectodermal nuclei, though in some embryos the evidence for this is scanty.

C. ANALYSIS OF MODIFICATION IN FIRST PHASE

The entire period of development from ½ hour to 6½ hours after laying is highly susceptible to KCN. Treatment of eggs for short periods results in grossly anomalous or in unorganized development if it is not prevented altogether.

Unorganized development is the result of a proliferation of cleavage cells within the egg, which have failed to become incorporated in the cortical cytoplasm.

Axial development is accomplished after light dosage in some cases. Such embryos are formed by the germ layers. Ectoderm is formed where cleavage cells have been incorporated into the cortical cytoplasm of the egg. It shows severe effects of KCN treatment. Mesoderm, formed from the injured superficial cells, shows little cytological injury.

Injury effects of treated eggs are apparent in the cortical cytoplasm and in the internal cytoplasm of the egg. They appear to be greatest on the ventral surface near the anterior part of the egg. Because of this injury of cortical cytoplasm, cleavage cells fail to become imbedded in it, and no blastoderm is formed. The cleavage cells which have not arrived at the egg periphery, on the other hand, continue to divide.

When cleavage cells become incorporated in the cortical cytoplasm, a continued development results in an axiate embryo. The more perfect of these lie on the ventral surface, while the dwarfed and badly deformed ones usually lie far laterally. Conversely, all cellular proliferation within the egg, away from the cortex, results in unorganized cell masses.

II. SECOND PHASE: 6½–12 HOURS

A. GENERAL SUSCEPTIBILITY

Developmentally this is the period in which the blastoderm is formed. All of the cleavage cells reach the cortical cytoplasm during the early part of the period; and by the latter part (12 hours) the cells on the ventral surface become crowded and columnar, to form the ventral plate. Visible axial development of this plate is then apparent.

During the early part of this phase, although the eggs are still undergoing blastokinesis and are visibly but slightly advanced over the eggs of the latter part of the first phase, they show a markedly different reaction to treatment with KCN.

In the first place the eggs are more resistant. This is shown, first, in that a longer period of treatment is required before they show evidences of treatment and, second, in that a much greater dosage is required to inhibit any further development (see Figs. 1*B* and 3). More than 15 minutes of treatment is required before any of the eggs show effects. From this time the curve rises somewhat slowly to a dosage of 45 minutes, then more rapidly to 60 minutes, after which 90 per cent of the eggs are killed. Seventy-five minutes of treatment is required to prevent any of the eggs from showing further development. Even after the effects of treatment become apparent, these are in contrast to those of the first phase in that all of the treated eggs show an axiate, organized development, though anomalous.

In the second place this phase is distinguished from the first by the type of terata produced. All of these are axiate, as mentioned above. The terata are elongate, twisted, and distorted. Their segmentation is deep and sometimes appears bilaterally asymmetrical. Cephalic lobes are well formed, and rudiments of mouth parts are present. The ventral ganglionic chain is apparent in some (Fig. 11).

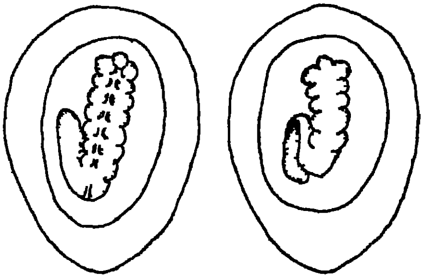


FIG. 11.—Terata of the second phase

For an understanding of this anomaly it is necessary to refer to normal development of the beetle embryo during the period of germ-layer formation. During this period the ventral plate, which has formed on the ventral egg surface by a thickening of the blastodermal cells, elongates posteriorly and flexes around the posterior extremity of the oöplasm and pushes dorsally and anteriorly until a caudal extension is formed which almost touches the cephalic lobes forming at the anterior extremity of the plate. The ventromedial cells of the ventral plate then become very tall, undergo mitotic nuclear division, and form the mesodermal layer internal to the ectodermal cells of the ventral plate.

The terata of the second phase are maldevelopments of the elongation of the ventral plate. Instead of flexing normally dorsoanteriorly and slightly to the right, they may flex in any direction. Since the plate is limited from extending farther posteriorly by the confines of the egg, it becomes displaced and distorted, twisted and turned. The normal dorsoanterior flexion appears to be inhibited, as few embryos succeed in completing this phase of development.

B. DIFFERENTIAL SUSCEPTIBILITY: HISTOLOGICAL

1. *Eggs incubated after treatment.*—Only one type of egg need be described here, since treated eggs capable of further development develop as organized axiate embryos.

In section the ventral plate of these embryos is usually narrower laterally than the normal, and is thinner vertically. There can be little doubt that the primary injury is to the ventral ectodermal plate. In the more severely injured, only a few small scattered nuclei remain. Cell outlines are obliterated, and occasional empty vacuoles are found. Strangely, the shape of the normal ectodermal layer is retained. This is thicker in the center and thinner laterally.

The mesodermal layer, normally a thin sheet of cells lying over the inner surface of the ectoderm, is here abnormally thick and abundant. It not only covers the inner surface of the ectoderm but extends farther laterally than the ectoderm. This layer is a cellular syncytium (Fig. 12).

The section illustrated also passes transversely through the tip of the caudal plate. Here no ectoderm is found, although the mesodermal mass appears to retain lateral prominences and a ventral groove. Mesoderm of the caudal plate is also shown to extend farther laterally than normal. This regulation embryo is largely a mesodermal embryo.

An egg showing extreme displacement of the embryonic plate laterally is shown in Figure 13. The ectoderm of this egg has shown a greater recovery than that of the former, and a prominent medial groove is present. Cellular debris is found in the ventral groove, indicating that the medial portion of the plate was either more severely injured or that it did not undergo recovery. The organization of ectodermal cells into two lateral masses indicates an attempt at formation of a ventral chain ganglion.

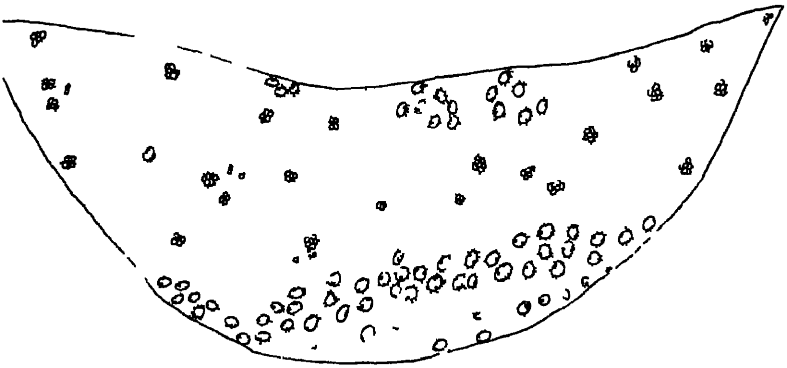


FIG 12.—Section through thoracic portion of second-phase embryo. Tip of caudal plate shown on dorsal (concave) side of egg Ectoderm almost absent in this region. Mesoderm hyperplastic

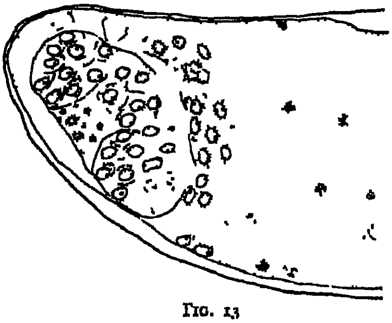


FIG. 13

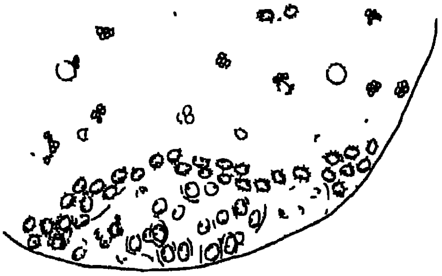


FIG. 14

FIG. 13.—Section of second-phase embryo in abnormal position. Ectoderm shows considerable recovery. Mesoderm hyperplastic.

FIG. 14.—Section of mid-thoracic region of second-phase embryo showing differential recovery in the ectoderm. Development in center of ectodermal layer is the first rudiment of the central nervous system, making a delayed appearance.

Mesodermal cells are abundant but at this level do not lie so closely applied to the ectodermal cells. The egg likewise shows extreme injury to the cytoplasmic cortex, and this injury becomes more pronounced medioventrally.

Differential recovery is illustrated in the ectoderm of some embryos (Fig. 14). The ectodermal cells of the embryonic plate are distinct medially and have formed a central ganglion-like structure which is in the first stages of development. Lateral to this the ectodermal cells are still very indefinite. Mesodermal cells have spread laterally beyond the limits of the ectoderm. Mesoderm is again much more abundant than it is in the normal embryo.

2. *Eggs not incubated after treatment.*—In eggs which are fixed, stained, and cleared just after treatment, the cleavage cells are seen at the egg surface. These are normally more numerous on the ventral surface just behind the point of the egg having the greatest vertical diameter. This is also the region at which the cleavage cells first reach the cortical cytoplasm. From here the concentration of cells diminishes over the remainder of the surface to the dorsal cytoplasm, where their concentration is least.

In eggs treated at 7 hours and fixed ½ hour after treatment is discontinued, injury is not so distinctly apparent as in those treated in the first phase. However, an area appears at the cell concentration center, where the cells appear less distinct than they do in normal eggs. This area extends forward into the presumptive cephalic lobe level and posteriorly along the medioventral line through the upper thoracic region. The entire area most affected is concentrated more along the median line than it is in first-phase eggs (Fig. 15).

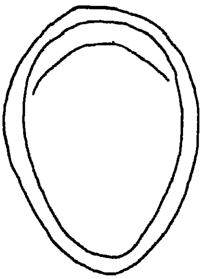


Fig 15

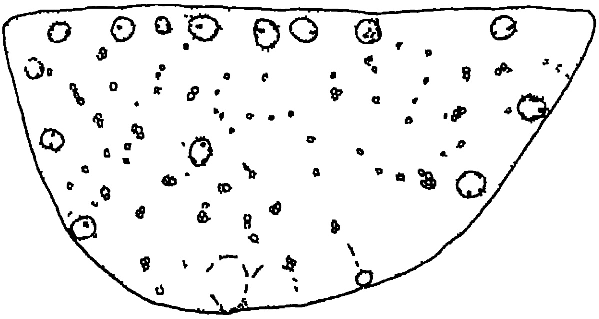


Fig 16

Fig. 15—Diagram of an egg of second phase showing differential susceptibility. Egg viewed from ventral surface.

Fig. 16.—Section through presumptive prothoracic level of an egg treated at 7½ hours. The differential susceptibility is shown in the medioventral blastoderm.

A study of sectioned eggs discloses the reason for the differences. Cleavage cells are well implanted in the cortical cytoplasm laterally and dorsally, but ventroanteriorly, where normally they are most numerous and best established, there are relatively few.

Coincident with the scarcity of normal cleavage cells there is a scanty cortical cytoplasm. In many eggs there are spots where it is almost, if not entirely, absent, and vacuoles appear under its former surface (Fig. 16).

Nearer the posterior egg extremity more cleavage cells appear in the cortex, but these are small and are scantily surrounded by cytoplasm. Indeed, the cytoplasm shows signs of great injury. It is the cytoplasm in all cases which appears to have been injured most by cyanide exposure, since nuclei with very little cytoplasm may be found in mitotic division.

C. SUMMARY AND ANALYSIS OF TREATMENT DURING SECOND PHASE

The second phase begins at 6½ hours and lasts until 12 hours at incubation temperature. It is recognized by the increased tolerance of the eggs to cyanide treatment and by the type of terata obtained after such treatment.

All terata of this phase are axiate embryos They are elongate and abnormally twisted

The embryos are composed of the germ layers Of these the ectoderm shows effects of severe injury, but also considerable recovery. Recovery, like injury, is differential within the embryonic plate, namely, in a mediolateral direction, and anteriorly and posteriorly from the region of highest susceptibility The internal layer or mesoderm is abnormally abundant and appears to be the dominant tissue of second-phase embryos It is formed, as in normal embryos, by the mitotic division of ectodermal cells, where the latter have survived

The type of anomaly is recognizable as a maldevelopment of a particular stage of normal development in which the embryonic plate grows in length In the terata this occurs abnormally, owing to an excessive amount of mesoderm and inhibition in the injured ectoderm. The embryos become abnormally twisted during elongation of the plate and remain in this condition because the normal contraction fails to follow.

III. THIRD PHASE: 12–16 HOURS

A GENERAL SUSCEPTIBILITY

In normal development this period is characterized by the thickening of the ventral blastoderm and the growth in length of the ventral plate thus formed. The plate flexes dorsoanteriorly at its posterior extremity and forms the caudal plate. Ventromedially the columnar cells of the blastoderm become tall By their mitotic division the mesodermal layer is formed as a thin lamina of cells lining the embryonic plate At the anterior extremity of the plate, and likewise at the extremity of the caudal plate, this internal layer becomes the entodermal rudiment. In the more advanced embryos of this stage the ectodermal cells of the medial portion of the embryonic plate of the thoracic region are proliferating to form the anlagen of the ventral ganglia. Posteriorly, development is less advanced, and here the ventral plate is still in the process of forming the mesodermal layer (Fig. 17).

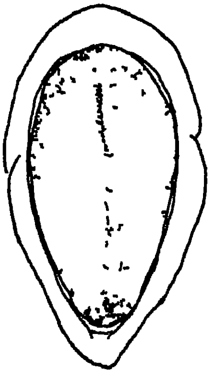


FIG 17

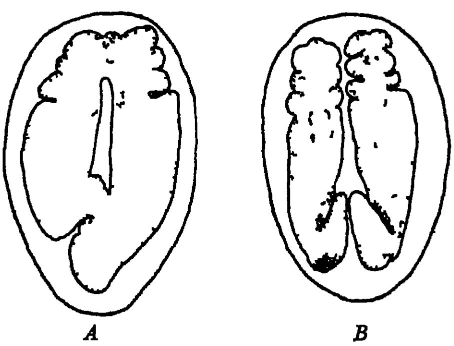


FIG 18

FIG. 17 —Egg of 16⅓ hours, showing thickening of ventral plate as a dark medial band The thickening is due to the formation of the mesentodermal layer

FIG. 18 —*A*, embryo of third phase, showing partial duplicity Doubling begins in prothoracic region and extends anteriorly into the head and posteriorly through the lower thorax *B*, twinning or complete separation and reduplication of bilateral parts.

During the third phase, eggs are more susceptible to KCN than they are in the second phase, and somewhat less so than they are in the first Complete inhibition of development is accomplished in 90 per cent of the eggs after from 50 minutes to 1 hour of treatment (see Figs 1*C* and 3)

Eggs treated for 20–40 minutes tend to produce a doubling of the ventral plate along the anteroposterior axis This is the characteristic anomaly resulting from treatment at this time, and has been encountered in some form in 80 per cent of some treated batches of eggs

Doubling (*duplicitas*) in these cases begins in and usually affects only the thoracic portion of the plate. In several cases it extends anteriorly to include the head, while in a few it likewise extends posteriorly and produces complete separation or twinning (Figs 18*A* and *B*)

A more complicated form of doubling is illustrated by the embryo shown in Figure 19 It represents *duplicitas anterior* and partial *duplicitas posterior* but is unpaired in the prothoracic region. This egg was treated at 15 hours and was therefore nearing the end of the phase. Determination of definite laterality had probably been realized in the prothoracic region at the time of treatment, and was therefore no longer subject to the same differential susceptibility as the remainder of the plate. Farther anteriorly in the head and farther posteriorly the levels were still labile, and were consequently affected in the characteristic manner. The lower thoracic level and the head level therefore show doubling, while the prothorax is undivided.

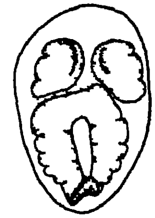


FIG 19 —Anterior and partial posterior duplicity in late third-phase embryo The prothoracic region, being precocious over other levels, has passed the labile stage and did not divide as did more anterior and posterior levels

B DIFFERENTIAL SUSCEPTIBILITY

Injury from the effects of cyanide treatment is apparent in the sections of embryos treated for 25 minutes Again this occurs in the medial portion of the ventral plate, in that region where the first concentration of cleavage cells occurred and which leads in general development From this area the effects spread anteriorly and posteriorly but remain concentrated along the medial portion of the plate. The particular cells most affected are those which are proliferating the ganglionic rudiments of the ventral chain of ganglia obliquely laterad from the median line. Many of the more lateral cells of the plate continue their activity, while the more medial ones become broken down. The impression obtained from this picture is that the center integrating these two slightly lateral areas is destroyed, permitting these regions to continue or resume development without this integration. The most noticeable effects are toward the anterior portions of the embryo, while the caudal plate is the last to suffer (Figs. 20 and 21).

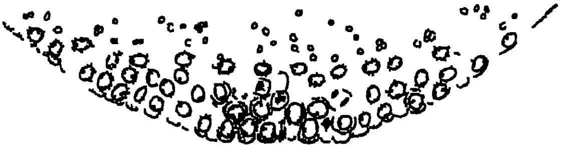


FIG 20 —Section through anterior thoracic level of treated third phase embryo. Most apparent injury in medial ectodermal cells

C. ANALYSIS

At the time of treatment (12–16 hours) the ventral plate is in the process of extension and elongation. In the most advanced embryos the central region of the ventral plate is showing the organization of paired centers, the ventral ganglia. Injury is then greatest where regional activity is highest, namely, in the medial portion of the ventral plate. When recovery occurs, centers just lateral to the median line become most active and develop independently of each other. A portion of the embryonic plate becomes organized around each of these centers and produces bifidity.

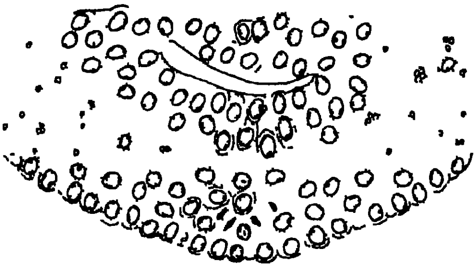


FIG. 21.—Same embryo as Figure 17. Section taken more posteriorly so as to include the basal portion of caudal plate. Injury is apparent in medial portion of ventral plate but is not noticeable in the caudal plate.

As in former phases, the prothoracic region is most highly susceptible. Doubling starts there and extends into the head and into the mesothorax secondarily. The prothorax likewise becomes determined bilaterally in advance of head and body levels.

DISCUSSION

Two evident features of this study are (1) that there are distinct phases during early development in which the reactions of eggs to cyanide treatment differ markedly, and (2) that characteristically distinct developmental modifications are produced in each phase by differential susceptibility of the egg to cyanide, indicative of physiological changes leading to the establishment of a morphological pattern.

SUSCEPTIBILITY PHASES

The distinct changes in susceptibility which occur during early development in *Bruchus*, as shown by Figures 1–3, do not coincide with visible steps in morphogenesis. It is probable that they do mark physiological processes initiating, and to some degree accompanying, developmental processes. For example, the first phase is found to coincide definitely with the period of embryonic determination in this insect (Brauer and Taylor, 1936). This is a physiological phase not delimited by distinct morphogenetic events. Each of the other phases probably also marks regional physiological differences which inaugurate morphogenetic processes.

In this connection it might prove of value to analyze groups of eggs by Bodine's (1929) method of measuring oxygen consumption in insect eggs, to determine whether respiratory differences actually exist during the phases indicated. Bodine (1934) found no significant differences in oxygen intake in grasshopper eggs during active development.

MODIFIED DEVELOPMENT RESULTING FROM DIFFERENTIAL SUSCEPTIBILITY

Bellamy (1919) showed that modifications produced by differential susceptibility of the frog's egg were the result of alterations produced in the physiological egg pattern. As the physiological pattern undergoes progressive developmental changes, cyanide treatment at different periods will be reflected by the different morphological patterns produced.

The same explanations account for the modifications obtained in the Bruchid egg. During the first 6½ hours of development, when the egg passes from an indeterminate to a determinate stage (Brauer and Taylor, 1936), with the exception of an anteroposterior polarity, pattern has not been entirely fixed. In Brauer and Taylor's paper, lability was indicated by the large number of double embryos and by the regulation embryos formed in the posterior two-thirds of the egg alone. In the present paper it is shown by the fact that, after only short periods of treatment, axial development is prevented in a large percentage of cases. Furthermore, this is the only period when cyanide treatment interferes with axiation.

The point is further emphasized herewith that the "lay" of the embryo becomes definitely established during the first phase. During this time the ventral region nearest the anterior surface, namely, the presumptive prothoracic-maxillary region, becomes the area of the first and most decided response to treatment. In this area cleavage cells first reach the cortical cytoplasm, and here they become most concentrated. It leads generally in morphogenesis. Differential susceptibility of this region may change the lay of the embryo laterally, although it maintains its general anteroposterior orientation.

The region of highest susceptibility in the bruchid egg corresponds rather closely to that of the libellulid egg designated by Seidel (1934) as the differentiation center. This conclusion was reached on the basis of constriction, and ultra-violet radiation experiments. Schnetter (1934), on the basis of constriction experiments on the honeybee egg, determined the organization center to be prothoracic-maxillary in location.

The cyanide experiments indicate a bearing on the integrating capacity of the region in the following manner. In eggs treated from 10 to 25 minutes, axial development is frequently inhibited; but in many instances there is still some regulative development of the egg. Such regulation embryos, instead of lying on the ventral surface, are centered around the anterolateral or anterodorsal cytoplasm. Thus, with the disintegration of anteroventral cortical cytoplasm, that of an adjacent region becomes dominant and asserts its more limited capacity to organize. The organizing capacity, like the gradient of differential susceptibility in this cytoplasm, is from the ventral toward the dorsal surface.

By the beginning of the second phase the axiate pattern and the general position of the embryo have become determined. Further alterations are now brought about by differential susceptibility and differential recoveries within this general pattern. The peculiar form assumed by these embryos is due to a modification of the relationships of the primary layers. The abnormal twisting which the embryos undergo is explainable on a mechanical basis, namely, that the inner mesoderm becomes more abundant and more active than the more affected ectoderm. The result of this is that normal flexure of the embryonic plate is prevented— and flexures may even be reversed.

By the modifications produced in the third phase it becomes evident that all parts of the embryonic plate are not yet irrevocably determined, at least as regards bilaterality. The modifications produced in the plate at this time range from partial doubling (*duplicitas cruciata*) to complete twinning. This implies that any or all levels of one side of the plate still have the capacity to reduplicate the other side. The reduplication as here produced is explainable in the sections of the embryonic plate. As shown, it is due to the greater susceptibility of the medial portion of the plate, and a subsequent physiological isolation of the more lateral portions, which assume a physiological dominance. Doubling begins at the most susceptible region, which is prothoracic. From here it extends forward into the head and posteriorly through the thorax. It may likewise spread farther anteriorly and posteriorly to produce complete separation and reduplication.

Differential susceptibility has here produced the same effects, after a fashion, obtained by Krause (1934) by mechanical means. By the removal of portions of the embryonic plate of the cricket, *Tachycines*, by puncture, or by incisions made on the plate with a glass needle, he produced various forms of reduplication. He found the type to be dependent upon the part of the plate from which removal of material was made, and upon the stage of development of the embryo. According to his figures, the embryonic plate of *Bruchus* is at approximately the same stage of development at 12–16 hours as that of *Tachycines* at the time the operations were made.

Krause has explained doubling by the mechanical separation of parts and the capacity of these parts to reduplicate because of the regulative capacities retained by the parts up to that particular stage of development. In *Bruchus* it is apparent that the physiological isolation produced by differential inhibition of oxygen intake produces the same effects that mechanical separation produce. The two insects appear to be in close agreement as to the progress of embryonic segregation within the embryonic plate, namely, from prothorax anteriorly and posteriorly.

It will be noted that doubling of the embryonic plate takes place at a different stage than it did after anterior cautery and anterior constriction in former experiments (Brauer and Taylor, 1936). At that time reduplication was obtained during the first phase. The duplicate embryos were always formed at more posterior levels than in the normal egg. In the present cases the embryos retain their natural level, as determined in earlier stages.

DIFFERENTIAL SUSCEPTIBILITY OF EMBRYONIC LAYERS

Certain impressions pertaining to origin of germ layers and their relative values may be obtained in these studies. It must again be pointed out that cyanide treatment during the first phase takes place prior to the arrival of cleavage cells in the superficial cortical cytoplasm. In these eggs it appears that the cleavage cells are less susceptible to treatment than the cortical cytoplasm. The cells continue to proliferate except after very severe treatment, but they do not react with the cortical cytoplasm. The result is that no blastoderm is formed. The masses of cleavage cells without a cortical cytoplasm are incapable of giving any kind of organized development under these conditions. It is thereby indicated that activity of cortical cytoplasm is necessary for the determination of prospective values; and furthermore, when the differential susceptibility of egg regions is taken into consideration, it appears to be the cytoplasm of the anteroventral region that is necessary for complete organized development.

In the second phase the eggs are subjected to treatment prior to the formation of a blastoderm, or, at any rate, before it has been completed. In eggs which continue to develop after treatment, an ectoderm is always formed—at least in part. Its cells quickly give rise to an internal layer which is the mesoderm, except in two definite areas, where it has been designated as entoderm. The inner layer, either because of its release from the physiological dominance of the injured ectoderm or because it actually absorbs some material from its injured cells, undergoes a hyperplasia. Some of the resulting embryos are preponderantly mesodermal embryos.

THE HEAD

It becomes readily apparent that in the sequence of physiological development of pattern in this insect the head is secondary in origin and remains so during the phases studied. From the works of Seidel, Schnetter, and Krause it is probable that this is the case in other insects. To what factors in development does thoracic dominance owe its origin? For how long during development is this dominance maintained? Surely the insect with its specialized anterior sensory development has a cephalic integrating center! These and other questions arise in this connection, but so far are only suggested.

SUMMARY

1. The development of pattern was studied in the egg of the cowpea weevil by an analysis of the modifications produced by the differential susceptibility of the egg to KCN.
2. In early development three metabolic phases are distinguishable. They are characterized by the degree of susceptibility and by the nature of the modifications produced.
3. The phases are as follows: First phase, from oviposition to 6½ hours after oviposition, during which time the eggs are highly susceptible. The terata of this phase are typically inaxiate. Second phase, from 6½ to 12 hours, during which time the eggs are much more resistant and the terata are elongate and always axiate when development takes place at all. Third phase, 12–16 hours, at which time the eggs are again less resistant than in the second period. The characteristic modification is partial to complete doubling along the anteroposterior axis.
4. During the first phase susceptibility becomes highest on the ventral surface. The most susceptible area gradually becomes restricted to the presumptive prothoracic-maxillary region.
5. Severe injury to the anteroventral cortical cytoplasm prevents its reaction with cleavage nuclei and their aggregation at that place, although the nuclei may continue to multiply within the oöplasm for some time. With destruction of anteroventral cytoplasm a modified embryonic plate may be formed around a new center or centers lying more dorsal. Capacity to form an organized axiate plate appears to become less the farther the new center is removed from the ventral surface.
6. Aggregations of cells are frequently formed by cleavage nuclei, which fail to become imbedded in cortical cytoplasm. These cells may undergo hyperplasia to form amorphic masses.
7. In second-phase eggs injury is most severe in the ectoderm of the embryonic plate. From the presumptive prothoracic-maxillary region injury extends in a declining gradient through the anterior portion of the head and posteriorly through the embryonic plate. Differential recovery frequently follows the same course.

8. Mesoderm (mesentoderm) is always formed in second-phase embryos. With the injury of ectoderm the mesoderm becomes hyperplastic. Its great increase is probably the cause of the abnormal flexions in these embryos.

9. Third-phase eggs are treated in the ventral-plate stage. Injury is severe in the medial portion of the plate anteriorly, and declines anteriorly and posteriorly. Differential recovery follows the same course.

10. Separation of the plate along the anteroposterior axis is easily accomplished by differential susceptibility. When this occurs, there is a reduplication of the other half, by each side. This is an indication that determination of definite bilaterality has not been accomplished at this particular time.

11. The prothoracic region is the first to become definitely determined as to bilaterality. After this occurs, doubling and reduplication are no longer possible in it, although head levels and lower body levels may still reduplicate.

12. The location of the integration center in *Bruchus* agrees with the location of the center in other insects which have been studied, but differs from other axiate animals in that it is not anterior in the head.

LITERATURE CITED

BELLAMY, A. W. 1919. Differential susceptibility as a basis for modification and control of early development in the frog. Biol. Bull., 37:312–61.

BODINE, J. H. 1929. Factors influencing the respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., 2:459–82.

———. 1934. The effect of cyanide on the oxygen consumption of normal and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., 4:397–404.

BRAUER, ALFRED, and TAYLOR, ALBERT CECIL. 1936. Experiments to determine the time and method of organization in bruchid (Coleoptera) eggs. Jour. Exper. Zoöl., 73:127–51.

CHILD, C. M. 1924. Physiological foundations of behavior. New York: Henry Holt & Co.

HYMAN, L. 1919. On the action of certain substances on oxygen consumption. II. Action of potassium cyanide on planaria. Amer. Jour. Physiol., 48:340–71.

KRAUSE, GERHARD. 1934. Analyse erster Differenzierungsprozesse im Keim der Gewächshausheuschrecke durch künstlich erzeugte Zwillings-Doppel- und Mehrfachbildungen. Roux' Arch. Entw'-mech., 131:285–323.

SCHNETTER, MARTIN. 1934. Physiologische Untersuchungen über das Differenzierungszentrum in der Embryonalentwicklung der Honigbiene. Roux' Arch. Entw'mech., 131:285–323.

SEIDEL, FRIEDRICH. 1929. Untersuchungen über das Bildungsprinzip der Keimanlage im Ei der Libelle Platycnemis pennipes. Roux' Arch. Entw'mech., 119:322–440.

———. 1934. Das Differenzierungszentrum im Libellenkeim. I. Die dynamischen Voraussetzungen der Determination und Regulation. *Ibid.*, 131:135–87.

———. 1935. Der Anlagenplan im Libellenei. *Ibid.*, 132:671–751.

ULTRA-VIOLET RADIATION OF GRASSHOPPER EGGS (ORTHOPTERA)[1]

(Seven figures)

O. MALCOLM RAY AND JOSEPH HALL BODINE
Zoological Laboratory, State University of Iowa

THE numerous contributions in the field of the biological action of ultra-violet light may be divided into four general groups based on the experimental materials used: first, the effects on protoplasm as studied in protozoa and plant cells; second, the effects on marine eggs; third, the bactericidal effect; fourth, the effects on chemical compounds of especial biological importance (Heilbrunn, 1932; Heilbrunn and Daugherty, 1933; Black, 1936; Lillie and Baskervill, 1922; Wyckoff, 1931; Crowe, 1936; Hollaender and Duggar, 1936; Harris, 1926; Spiegel-Adolf and Oshima, 1929; Laurens, 1928; Clark, 1922; Burge, Fischer, and Neill, 1916).

In the present work a quantitative study has been made of the effects of ultra-violet irradiation of the developing eggs of the grasshopper, *Melanoplus differentialis*. This material is especially suitable for use in an investigation of this kind since the course of normal development is well understood (Slifer, 1932*a*) and various physiological considerations are at hand (Bodine, 1929, 1932; Bodine and Boell, 1934; Slifer, 1932*b*).

MATERIAL AND METHODS

The effects of ultra-violet light have been determined largely through changes in the oxygen consumption of the eggs. For this work, modified Krogh differential manometers were employed (Bodine, 1929). Five to ten eggs were used in each manometer, and experiments were performed in such manner that different eggs were used daily as well as the same eggs repeatedly. One hundred or more eggs were used in obtaining data for each oxygen-consumption curve, and from two hundred to one thousand eggs constituted an experiment. Hatching records were also kept.

As a source of ultra-violet light the entire spectrum of an air-cooled quartz mercury-vapor lamp operating on 110–15 volts A.C. and 3.7 amperes at a distance of 15 cm. was used. The eggs were radiated on damp filter paper, in glass dishes covered with quartz plates 2½ mm. thick, or with glass plates 1 mm. thick for experimental and control experiments, respectively. The dishes were placed on wet toweling, and their temperature was kept at 22°–24° C. during the irradiation. The dosage was controlled by varying the length of exposure.

Removal of the chorion was accomplished by placing the eggs on adhesive tape, loosening the chorion with a needle, and then removing the egg, leaving the chorion behind on the tape.

RESULTS

1. *The permeability of egg membranes to ultra-violet light.*—The grasshopper embryo and yolk are surrounded by two membranes during the greater part of embryonic development (Slifer, 1932*a*). When the egg is laid, only the outer membrane, the chorion,

---

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

is present. About the seventh day of development the inner transparent cuticle is laid down, after completion of which the outer membrane (chorion) may be peeled off, permitting direct observation of the embryo. It was first necessary to determine the permeability of these membranes to ultra-violet light.

To test for the penetration of primary radiations, a lithopone paste (Ellis and Wells, 1925) was put between two quartz plates; and pieces of the membranes, moist and dry, were placed on the plates and exposed to ultra-violet light for various intervals up to 2 hours. During this time there was no darkening of the lithopone paste beneath the pieces of chorion and cuticle, indicating an impermeability to the primary radiations of ultra-violet light.

TABLE 1

EFFECT OF VARIED AMOUNTS OF ULTRA-VIOLET RADIATION ON GRASSHOPPER EGGS WITH CHORION AND CUTICLE AND WITH CHORION ALONE

Prediapause eggs have only the chorion for 7 days after laying; beyond that time both membranes are present. The values are given in percentage of eggs started and the days after diapause on which the eggs hatch are indicated.

TIME OF RADIATION		DAYS POSTDIAPAUSE ON WHICH EGGS HATCH							
		17	18	19	20	21	22	23	Total
Prediapause eggs:									
One hour daily for 5 days from laying	Control	0	0	80	2	6	0	1	89
	Experimental	0	3	88	1	0	1	0	93
One hour daily for 10 days from laying	Control	0	2	80	5	1	0	0	88
	Experimental	0	3	78	0	1	1	2	85
One hour daily for 15 days from laying	Control	0	0	86	4	0	0	0	90
	Experimental	0	0	82	2	3	3	0	90
Diapause eggs:									
One hour daily for 28 days	Control	1	0	88	1	3	1	0	94
	Experimental	2	2	87	0	5	0	0	96
Postdiapause eggs:									
One hour daily for 20 days		5	23	20	37	5	0	3	93
Two hours daily for 20 days		8	12	34	34	4	0	2	94
Three hours daily for 20 days		6	8	28	32	6	0	10	90
Four hours daily for 20 days		12	20	40	14	6	0	0	92
Control—2 hours daily for 20 days under glass		0	16	14	38	10	0	6	84

The fact that only the chorion is present for the first 6 days after the eggs are laid and that it may be removed after the fifteenth day, leaving only the cuticle, provides an excellent opportunity to study the penetration of the secondary radiations as shown by effects on the eggs. The results of irradiation with the chorion alone and with both the chorion and cuticle are presented in Table 1. The upper part of the table represents a period during the first 6 days, when the chorion alone is present. In the remainder of the table both membranes are in place. It is apparent from this table that there is no effect of the irradiation on the intact egg. In the case of postdiapause eggs that were irradiated as much as 4 hours daily for 20 days, there is still no effect.

The oxygen consumption of prediapause and postdiapause eggs after irradiation is presented graphically in Figure 1. From inspection of this figure it may be noted that the respiration of the experimental and control groups is very similar, there being no obvious effect of the ultra-violet irradiation. In view of these results the chorion has been removed from all of the eggs used in the following experiments.

2. *The effect of single exposures on dechorionated postdiapause eggs.*—Figure 2 is a graphic presentation of the changes in oxygen consumption of groups of dechorionated

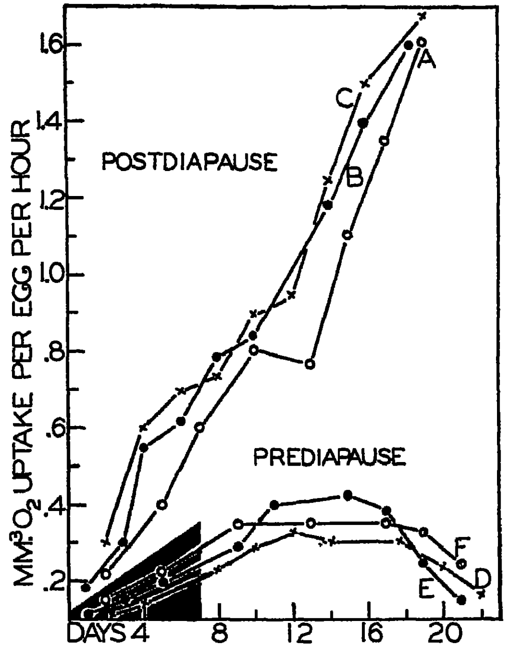


FIG. 1.—Effect of ultra-violet irradiation of prediapause and postdiapause eggs with chorion, and with chorion and cuticle present, on their oxygen consumption. In the postdiapause group, curve *A* (control) was irradiated 2 hours daily under glass, curve *B* was irradiated 2 hours daily, and curve *C* 4 hours daily. The eggs hatched at the end of this time. In the prediapause group, curve *F* is the control (2 hours daily under glass), curve *E* was irradiated 2 hours daily, and curve *D* 4 hours daily. The solid black area indicates the time during which only the chorion is present. The decreased respiration about the twentieth day is due to the normal occurrence of diapause.

eggs irradiated by single exposures of different lengths on the first day of postdiapause development. It should be noted that single exposures of less than 15 minutes produce no apparent effect. A 15-minute exposure causes a small but consistent stimulation, while there seems to be a very definite threshold between a 15- and 30-minute exposure, the latter being decidedly detrimental to the eggs. Partial recovery is evidenced after exposures of 30 and 60 minutes, but longer periods of irradiation permit practically no development. The hatching record of these eggs is shown in Table 2. It may be seen that this is in agreement with the altered oxygen consumption and shows the maximum effect in 90-minute exposures. The curious retardation in hatching of eggs irradiated 30 minutes or longer reappears more strikingly in a later experiment.

3. *The effect of daily irradiation of postdiapause eggs.*—From an inspection of Figure 3 it may be noted that postdiapause eggs are particularly susceptible to short, daily ex-

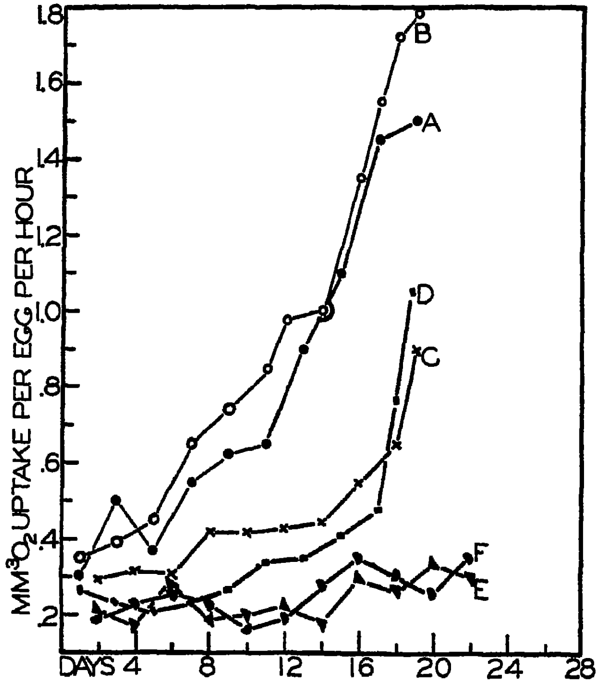


FIG. 2.—Effect of single ultra-violet irradiations on the respiration of dechorionated postdiapause eggs. The eggs were irradiated on the first day out of diapause, as follows: curve *A* (control) 2 hours under glass, curve *B* 15 minutes, curve *C* 30 minutes, curve *D* 60 minutes, curve *E* 90 minutes, and curve *F* 120 minutes.

TABLE 2

EFFECTS OF SINGLE ULTRA-VIOLET RADIATIONS OF THE AMOUNTS INDICATED ON THE FIRST DAY AFTER DIAPAUSE ON THE HATCHING OF DECHORIONATED EGGS

Values are given in percentage of eggs started and the days after diapause on which the eggs hatch are indicated.

TIME OF RADIATION	DAYS TO HATCH															
	21	22	23	24	25	26	28	30	31	32	34	37	43	47	50	To-tal
Control	32	30	22	4	....	....	....	...	....	....	....	....	....	....	....	78
15 minutes	22	30	14	10	4	....	....	....	....	....	....	....	....	....	....	80
30 minutes	....	8	18	6	2	2	6	....	....	4	4	....	2	....	....	52
60 minutes	....	....	....	6	....	6	6	6	6	4	4	....	....	....	....	38
90 minutes	....	....	2	2	....	....	2	2	8	....	2	4	....	....	2	24
120 minutes	....	....	2	6	....	2	4	2	6	....	....	....	4	....	4	30

posures to ultra-violet light Daily exposures of 5 seconds seem to have no effect. Fifteen-second exposures produce a slight stimulation in oxygen consumption but with a reduced hatch. Daily irradiations of 1 minute or longer greatly reduce all activity of the eggs, and only a few hatch.

4. *Recovery and increasing resistance with age.*—To study the recovery after various amounts of irradiation, ten groups of eggs were selected, and nine of these were given daily irradiations of 15 minutes for from 1 to 9 days, beginning on the first day of postdiapause development. Figure 4 is a graphic presentation of the changes in the oxygen consumption in these groups. After one or two exposures a slight stimulation is suggested. Three, four, and five 15-minute exposures permit development to about 50 per cent of normal. Six or more exposures, totaling 90 minutes or more, gradually retard development, and finally the respiration begins to decrease. Less than 5 per cent of the eggs hatch. The hatching record of this series is summarized in Table 3. Again, the group showing a slight stimulation in respiration shows also a marked decrease in hatch as compared to the control group. An even more decided retardation in hatching is demonstrated here than in Table 2.

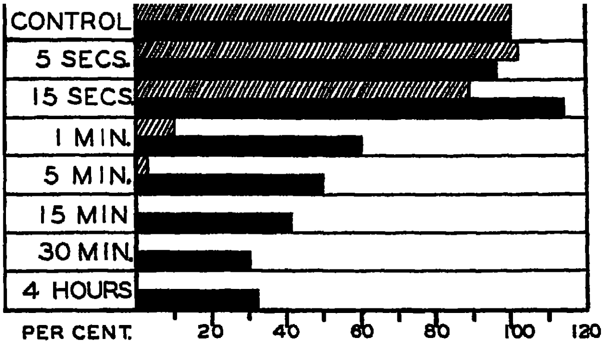


FIG. 3.—Effect of daily irradiations of amounts indicated for 18 days on the hatch and respiration of dechorionated postdiapause eggs. The diagonal-striped columns represent the percentage of hatch, and the solid columns represent the percentage of oxygen consumption, based on the controls as 100 per cent.

The resistance of the eggs to ultra-violet irradiation seems to depend on their physiological condition rather than simply to increase with age. Figure 5 summarizes the results with eggs that were given single 30-minute exposures at different stages of postdiapause development and indicates at first a moderate resistance to irradiation which decreases markedly by the sixth day and from that time increases with age to a maximum about the eleventh day. The hatching record was in close agreement with these changes in oxygen consumption.

5. *The differential effect on the eggs.*—It should be considered whether the effect is the same on the entire egg or whether it is limited to the yolk or to the embryo. In this series of experiments the eggs were partially dechorionated so that only the yolk was exposed. However, during yolk engulfment the embryo would become exposed about the tenth day of postdiapause development. The results are presented graphically in Figure 6. Two groups of eggs were prepared as above described and were irradiated 1 hour daily from the first day of postdiapause. Just before the embryos became exposed,

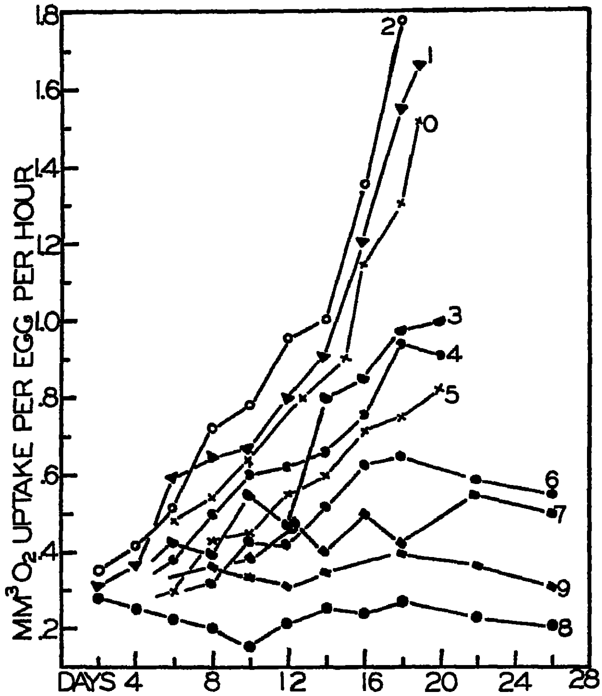


FIG. 4.—Effect on the respiration of dechorionated postdiapause eggs from one to nine daily irradiations of 15 minutes. The number of the curve indicates how many 15-minute irradiations a group has had over an equal number of days, i.e., curve *0* is the control group, curve *1* was irradiated 15 minutes on the first day out of diapause, curve *2* was irradiated 15 minutes daily on the first 2 days out of diapause, etc.

TABLE 3

EFFECT OF 15-MINUTE, DAILY ULTRA-VIOLET RADIATIONS FOR 1 TO 9 DAYS AFTER DIAPAUSE ON THE HATCHING OF DECHORIONATED EGGS

The values are given in percentage of eggs started and the days after diapause on which the eggs hatch are indicated.

No. of 15-Minute Radiations	Days to Hatch																
	17	18	19	20	21	22	24	26	28	31	32	33	37	40	43	65	Total
0	....	30	18	26	10	6	....	....	....	....	....	....	....	....	....	....	90
1	2	22	10	14	10	4	18	....	....	....	....	....	....	....	....	....	80
2	2	8	8	20	10	2	2	4	....	....	2	....	....	....	....	....	58
3	....	....	....	16	18	....	14	....	6	....	....	....	....	....	....	....	54
4	....	....	....	2	14	1	16	....	....	....	....	....	....	....	....	....	33
5	....	....	....	....	2	2	16	....	10	2	2	....	....	....	....	....	34
6	....	....	....	....	....	....	6	2	....	2	2	4	....	....	....	....	16
7	....	....	....	....	....	....	....	....	4	....	....	....	....	....	....	4	8
8	....	....	....	....	....	....	....	....	....	....	....	....	....	2	2	....	4
9	....	....	....	....	....	....	....	....	....	....	....	....	2	2	....	....	4

the irradiation of one group was continued under glass (controls), while the other group was exposed under quartz, as before. Apparently there is no effect on the respiration

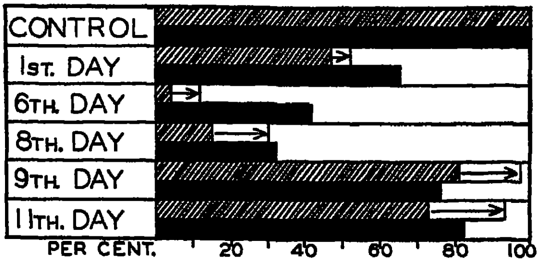


Fig. 5.—Effect of single exposures of ultra-violet light on the different ages of postdiapause eggs. The eggs were irradiated for 30 minutes on the day of postdiapause development, as indicated. The diagonal-striped columns, plus the portion occupied by the arrows, show the percentage of hatch. The solid black columns show the percentage of respiration over the entire postdiapause period. The portions occupied by the arrows show the percentage that died immediately after hatching. Percentages are calculated on the basis of the controls as 100 per cent.

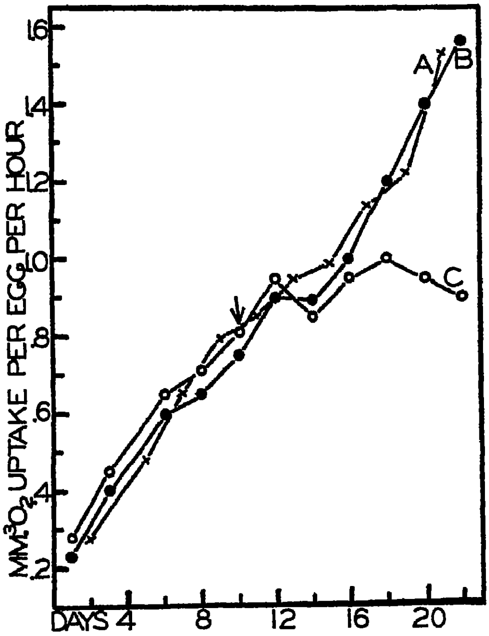


Fig. 6.—Effect of daily irradiations of 1 hour on the yolk of postdiapause eggs. Curve *A* (controls) was irradiated under glass. Curve *B* was irradiated with the yolk exposed until the tenth day(indicated by the arrow), after which it was also irradiated under glass. Curve *C* was irradiated during the entire period, with the embryo becoming exposed about the twelfth day.

until the embryo itself is exposed to the ultra-violet light, at which point there is a retardation and finally a decrease. Eggs in which only the yolk was exposed continued a normal respiration and hatched satisfactorily in contrast to irradiated embryos.

6. *The effect of ultra-violet light on the respiratory mechanism.*—Effects thus far presented have been obtained with developing eggs. It seems possible that the inhibited respiration may have been produced through effects on growth and metabolism rather than on the respiratory mechanism itself. Diapause (inactive) eggs and developing ones whose activity was temporarily and reversibly inhibited by keeping them at 5° C. were thought to offer an excellent opportunity to determine whether the ultra-violet light affected the respiratory mechanism primarily or through effects on growth, tissue structure, etc. The oxygen consumption of such eggs after irradiation is presented in Figure 7. It should be noted that in both types of eggs there was no apparent effect on the respiration while they remained inactive. None of the irradiated eggs hatched. Those that were kept at 5° C. showed a slight response when returned to developmental temperatures, and then degenerated. Such behavior tends to indicate that the effect of the ultra-violet light on respiration is produced through some intermediate factor rather than primarily on the respiratory mechanism itself. These results are essentially in agreement with those of Boell, Ray, and Bodine (1937), indicating that effects of X-radiation on the respiration of *M. differentialis* reported by Evans (1934) were perhaps produced in this indirect manner.

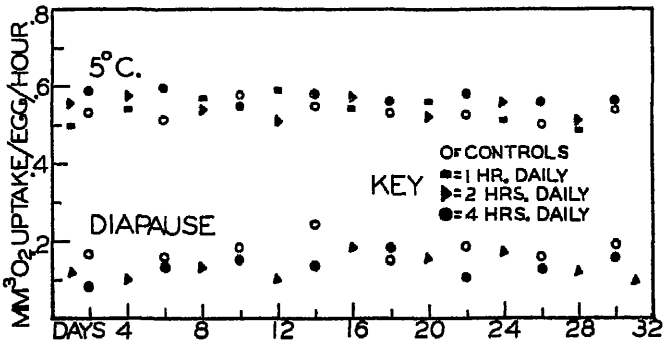


FIG. 7.—Effect of ultra-violet irradiation on the respiration of normally and experimentally blocked eggs. Four groups of eggs blocked experimentally by keeping at 5° C. are represented as follows: controls, irradiated 2 hours daily under glass; and three other groups, irradiated 1, 2, and 4 hours daily, respectively. These were 6-day postdiapause eggs. Three groups of normally blocked (diapause) eggs are shown: controls, irradiated 2 hours daily under glass, and two experimental groups, irradiated 2 and 4 hours daily, respectively. The groups are indicated in the key.

DISCUSSION

In view of the fact that the ultra-violet light does not penetrate the cuticle directly, it is to be assumed that any effects were produced by secondary radiations given off by the cuticle and that these radiations were of wave-lengths longer than those applied directly to the eggs, as is usually true when radiations are absorbed and given off. Presumably, then, the effective radiations were from the region of the long ultra-violet. This region has been found to be particularly active physiologically (Duggar, 1936).

Stimulated oxygen consumption was observed in several instances (Figs. 2, 3, and 4), but in each case it was accompanied by a reduced hatch. This is thought to demonstrate that the stimulation itself was due to some physiological disruption rather than simply to an increase in rate of the normal metabolic functions, and hence is correlated with a detrimental effect, appearing as lowered hatching percentages. Heilbrunn (1928), in studying the effects of ultra-violet light on *Amoeba*, observed a "clotting" of the protoplasm in connection with stimulation. Aside from those short exposures that produced an increased oxygen consumption, the inhibition of respiration and the decreased percentage hatch is proportional to the length of the exposure up to exposures of 2 hours, at which point a maximum effect is produced. It is further to be noted (cf. Figs. 2 and 3) that short, frequent irradiations are more effective than the same total exposure in a single irradiation. That the stimulation and inhibition occur simultaneously or are closely associated is indicated (Fig. 3) by the fact that increasing the irradiation as little as 45 seconds daily changes the results from the former to the latter.

It is generally considered that embryonic tissues become less susceptible to the action of radiation with increasing age, but various irregularities have been observed. Hinrichs (1925) found that the eggs of *Fundulus heteroclitus* were differentially susceptible to ultra-violet irradiation even in the first few minutes after fertilization, and that the regions of highest metabolic activity were most affected. Bachem and Dushkin (1935) report that the susceptibility of bacteria to ultra-violet irradiation is extremely dependent on their physiological condition. Similar results have been found with the grasshopper embryo in a study of its susceptibility at different ages (Fig. 5). Apparently there is a marked decrease in resistance to irradiation on the sixth day of postdiapause development. This period includes the completion of blastokinesis and the beginning of yolk engulfment, involving considerable activity on the part of the embryo. It has also been observed that the great majority of the embryos that are irradiated before this time and never hatch have ceased development at this point. It seems to be a period of extreme susceptibility. First-day eggs are only slightly more affected than 9-day ones. From the sixth day the resistance increases with age to a maximum about the fifteenth day.

A notable retardation in hatching has been demonstrated as an effect of ultra-violet irradiation of grasshopper eggs (Tables 2 and 3). Extreme variation in individual susceptibility is also indicated. It is a question of some interest why, if those eggs are going to hatch, some of them should hatch from 30 to 45 days later than the control groups. Similar effects, but to a lesser degree, have been noted by Nebel, Harvey, and Hollaender (1937), who found a marked inhibition in cleavage after irradiation of *Arbacia* eggs with the long ultra-violet. Unfertilized eggs could be activated to cleavage, which was also retarded.

In view of the fact, demonstrated by Bodine and Boell (1936), that the marked increase in oxygen consumption immediately following diapause occurs primarily in the yolk cells and extra embryonic material and that the yolk components constitute about 30 per cent of the total egg respiration at this time, the complete resistance of these components to ultra-violet irradiation (Fig. 6) is of considerable interest. Comparatively heavy daily exposures of yolk to ultra-violet light for 10 days, including this period of increased activity, show no apparent effect. This resistance is even more interesting when the presence of tyrosinase is considered. Bodine and Boell (1935) have shown that comparatively large amounts of tyrosinase are present in the grasshopper eggs and that practically all of it is in the yolk and its components. Narayanamurti and Ayyar (1929) report the activation of tyrosinase by ultra-violet irradiation; and Hinrichs (1925) believes that tyrosine acts as an optical sensitizer, rendering cells susceptible to the toxic action of ultra-violet light. Keeping these considerations in mind, it seems remarkable that the yolk and its components show no effects of irradiation.

SUMMARY AND CONCLUSIONS

Neither the chorion nor the cuticle around the egg of the grasshopper, *M. differentialis*, are permeable to direct ultra-violet radiations. The cuticle seems to permit the passage of effective secondary irradiations.

Short irradiations with ultra-violet light produced increased respiration and decreased hatch. Longer irradiations inhibit both respiration and hatching in amounts proportional to the radiation. Below the maximum effect a given amount of ultra-violet radiation is more effective in short frequent exposures than in a single one.

Variations in susceptibility to ultra-violet irradiation are correlated with physiological activity rather than with the chronological age of the embryo. Periods of increased activity are most susceptible.

An extreme variation in individual sensitivity and a marked retardation in hatching have been demonstrated.

The action of ultra-violet light has been shown to be on the embryo rather than on the yolk or its components.

Altered respiration is apparently produced through changes in growth and metabolism and not by action of the radiation directly on the respiratory mechanism.

LITERATURE CITED

BACHEM, A., and DUSKHIN, M. A. 1935. A study of bacterial sensitivity to ultraviolet radiation. Biol. Bull., 69:109–25.

BLACK, W. A. 1936. Experimental modifications of the effect of ultraviolet on protoplasm. I. *Ameba proteus*. Univ. Calif. Pub. Zoöl., 41:75–100.

BODINE, J. H. 1929. Factors influencing the rate of respiratory metabolism of a developing egg. (Orthoptera). Physiol. Zoöl., 2:459–82.

———. 1932. Hibernation and diapause in certain Orthoptera. II. Response to temperature during hibernation and diapause. *Ibid.*, 5:538–48.

BODINE, J. H., and BOELL, E. J. 1934. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., 5:97–113.

———. 1935. Enzymes in ontogenesis (Orthoptera). *Ibid.*, 6:263–75.

———. 1936. Respiration of embryo versus egg. *Ibid.*, 8:357–65.

BOELL, E. J.; RAY, O. M.; and BODINE, J. H. 1937. Effect of X-rays on the oxygen consumption of embryonic cells. Radiology, 29:533–40.

BURGE, W. E.; FISCHER, W. R.; and NEILL, A. J. 1916. The destruction of hormones, proenzymes and enzymes by ultraviolet radiation. Amer. Jour. Physiol., 40:426–32.

CLARK, J. H. 1922. The physiological action of light. Physiol. Rev. 2:277–309.

CROWE, M. O'L. 1936. The ultraviolet absorption spectrum curve of phthiocol, a pigment of the human tubercle bacillus. Jour. Biol. Chem., 115:479–84.

DUGGAR, B. M. 1936. Biological effects of radiation. New York: McGraw-Hill.

ELLIS, CARLTON, and WELLS, A. A. 1925. The chemical action of ultraviolet rays. New York: Chem. Catalogue Co.

EVANS, T. C. 1934. Respiration and visible development of individual grasshopper eggs following X-irradiation. Physiol. Zoöl., 7:556–71.

HARRIS, D. H. 1926. Observations on the velocity of the photo-oxidation of proteins and amino-acids. Biochem. Jour., 20:288–92.

HEILBRUNN, L. V. 1928. The colloid chemistry of protoplasm. Berlin: Gebruder Borntraeger.

———. 1932. Action of ultraviolet light on amoeba protoplasm. Collecting Net, 7:57–59.

HEILBRUNN, L. V., and DAUGHERTY, KATHRYN. 1933. The action of ultraviolet rays on amoeba protoplasm. Protoplasma, 18:596–619.

HINRICHS, MARIE A. 1925. Modification of development on the basis of differential susceptibility to radiation. I. *Fundulus heteroclitus* and ultraviolet radiation. Jour. Morph. and Physiol., 41:239–63.

HOLLAENDER, A., and DUGGAR, B. M. 1926. Irradiation of plant viruses and of microörganisms with monochromatic light. III. Resistance of the virus of typical tobacco mosaic and *Escherichia coli* to radiation from λ 3000 to λ 2250 A. Proc. Nat. Acad. Sci., 22:19–24.

LAURENS, HENRY. 1928. The physiological effects of radiation. Physiol. Rev., 8:1–91.

LILLIE, R. S., and BASKERVILL, MARGARET L. 1922. The action of ultraviolet rays on starfish eggs. Amer. Jour. Physiol., 61:57–71.

NARAYANAMURTI, D., and AYYAR, C. V. R. 1929. Studies in enzyme action. IV. Tyrosinase I. Jour. Indian Inst. Sci., 12:109–29.

NEBEL, B. R.; HARVEY, E. B.; and HOLLAENDER, A. 1937. The cytology of *Arbacia punctulata* activated by monochromatic ultraviolet radiation. Biol. Bull., 73:365–66.

SLIFER, E. H. 1932*a*. Insect development. III. Blastokinesis in the living grasshopper egg. Biol. Zentralbl., 52:223–29.

———. 1932*b*. Insect development. IV. External morphology of grasshopper embryos of known age and with known age and known temperature history. Jour. Morph., 53:1–21.

SPIEGEL-ADOLF, M., and OSHIMA, Z. 1929. Physikalisch-chemische Untersuchungen an bestrahlten Proteinen. VI. Mitteilung weiter Studien zum spektroskopischen und biologischen Nachweis von lichtner Änderungen an Proteinen. Biochem. Zeitschr., 208:32–44.

WYCKOFF, RALPH W. G. 1931. The killing of colon bacilli by ultraviolet light. Jour. Gen. Physiol., 15:351–61.

THE RATE OF OXYGEN CONSUMPTION OF FROGS DURING EMBRYONIC DEVELOPMENT AND GROWTH

(Six figures)

MEYER ATLAS
Department of Zoology, Columbia University

THE rate of oxygen consumption during development is an important factor in any analysis of the unknowns of metabolism responsible for development. For this reason many workers have attacked this problem on different species. Warburg (1908), with his work on the sea-urchin egg, was one of the first to achieve results that may be considered to approach quantitive accuracy. In 1915, with his well-known respirometer, he showed that during the development of the sea-urchin egg the respiratory rate (measured in cubic millimeters of oxygen consumed per hour) rose continuously up to gastrulation, which was as far as these observations went.

Bataillon (1896) might be considered the pioneer in this field on amphibian development. He measured carbon dioxide given off rather than oxygen consumed. Bataillon's data indicated the presence of peaks and troughs in the rate of respiration at various stages of development. Godlewski (1900) was the first actually to determine the rate of oxygen consumption of amphibian eggs. His data indicated a continuous increase in the rate. These earlier workers were handicapped by the lack of sufficiently accurate methods. Thus, Godlewski could only measure the rate of increase per day. The important matter from a physicochemical analysis of development is the instantaneous rate of oxygen consumption. An approach to the determination of this was made by Bialaszewicz and Bledowski (1915), who used the Winterstein microrespirometer on eggs of *Rana temporaria (fusca)*. With this apparatus the oxygen uptake can be determined for intervals as low as 15 minutes. By repeated observations an analysis of the instantaneous rate may be made. They concluded that there is a gradual rise in rate of oxygen consumption from fertilization until 150 hours later (when the external gills had reached their greatest development, and when a plateau had been reached in the curve relating rate of oxygen intake to time). Their data suffered, however, from observations which were too widely spaced. In 1921 Parnas and Krasinska attacked the problem anew by means of the Barcroft-Warburg respirometer. Their material included *R. temporaria·(fusca)*, *R. esculenta*, and *Bufo vulgaris*. Instead of a gradual increase in rate, these workers found that the rate of oxygen consumption increased abruptly at the onset of three critical stages: gastrulation, neurulation, and appearance of external gills. Between the beginning of these stages the rate seemed to remain constant. Owing to the temperature (15° C.) at which these investigators worked, and also to the small quantity of eggs used, they determined the total oxygen consumed over a lengthy period, and calculated the rate indirectly.

Such was the conflicting status of the question when the work on which this paper is based was begun in 1934. At the time, no data were present for American amphibians. It was considered important to reinvestigate this problem as a part of a general attack on the various factors in the rate of development, especially as affected by temperature.

Since the beginning of the work, several additional papers have appeared which are relevant to the subject: Brachet (1934) on *R. fusca*, Wills (1936) on *R. pipiens*, and Stefanelli (1937) on *R. fusca*. None of these is directly concerned with the question of change of instantaneous rate with time at constant temperature. The observations of the first two are too widely spaced to determine the answer to the question of the existence of critical periods. The last paper only considers fluctuations in rate during early cleavage.

It is hoped that the data presented by this paper will aid in the elucidation of this problem. At the same time, an attempt has been made to relate the changes in rate of oxygen consumption to other variables during development, including length and weight. Studies on the growth of the amphibian embryo have been made since Davenport (1897) and Schaper (1902). Until now, however, no attempt has been made to relate embryonic growth and rate of respiration. Although no causative interrelation need be assumed, still it is important to know how these phenomena vary simultaneously. Such data should be helpful in answering the question whether the increase in the respiratory rate is due to an increase in the intensity of respiration of the separate cells or whether it is due to an increase in the number of cells.

MATERIAL AND METHODS

The eggs of *R. pipiens* were obtained after injection of anterior pituitary gland by the method described by Rugh (1937). They were fertilized artificially and kept in finger bowls until used in the experiments. In the earlier experiments the material used was timed according to stage from the data given by Atlas (1935). In the later experiments each batch of eggs was kept in an incubator at 20° C. ± 0°.5. Care was taken to avoid crowding, and the average density was not greater than 50 eggs for 200 c.c. of water (pre-gastrula stages) and 25 eggs for 200 cc. (later stages).

In the earlier experiments Great Bear spring water was used as the medium for the eggs and embryos. In later experiments an artificial pond water was made by diluting Ringer's solution with nine volumes of water. Results with this one-tenth Ringer's solution did not give any appreciable differences from that with the natural spring water.

Two types of apparatus were employed in obtaining the measurements of oxygen consumption. In the earlier experiments the Barcroft-Warburg manometric respirometer was employed. A recent outline of the theory of this apparatus is available in Dixon (1934). Two sizes of vessels were employed: a small one (volume, 8–10 cc.), which produced a change of 1 mm. in the height of Brodie's fluid for every 0.65–0.75 cu. mm. of gas consumed; and a larger one (volume, 24.5–27.0 cc.), which produced a change of 1 mm. in the height of fluid of the manometer for every 1.97–2.1 cu. mm. of gas consumed. A large number of observations was obtained with the Fenn-Winterstein volumetric respirometer, as described by Fenn (1927). The blank vessels of this apparatus were approximately 15 cc. in volume. Each millimeter excursion of kerosene drop corresponded to an uptake of approximately 0.4 cu. mm. of oxygen.

The number of eggs used in each flask varied for different stages studied. For stages up to gastrulation, 50 eggs were employed. In later stages fewer embryos were needed, and at the time of operculum formation 5 tadpoles were sufficient. It was difficult to remove the embryos from the surrounding membranes before the closure of the neural folds. It was found expedient, before this stage, to remove as much of the jelly as was possible, by placing the eggs on a wet towel or on filter paper and cutting into the innermost layer of jelly with scissors. This leaves a small amount of jelly adhering to the vitelline membrane which, however, does not appear to affect the oxygen uptake.

Attempts were made to remove the jelly by means of 0.7 per cent KCN. This method was first described by Bataillon (1912) and was used with success on *R. fusca* by Brachet (1934). If *R. pipiens* eggs are placed in this solution at the optimum period (which is about 30 minutes after fertilization), much of the jelly is dissolved within 45 minutes; but only perhaps 10 per cent of the eggs will continue to develop normally in favorable batches, even after prolonged washing (up to 2 hours). Such normally developing eggs, however, do not differ in their oxygen consumption from those which retain small amounts of jelly which have not been exposed to KCN. This confirms Brachet (1934). The difference in behavior to KCN is probably a species difference.

The procedure finally adopted in preparing the experiment was the following: Jelly was removed when present. Observations were then made regarding stage and size. The latter was determined, for the early stages (up to 5 mm.), as the greatest diameter or length, by means of an ocular micrometer in a compound microscope. For lengths greater than 5 mm. a millimeter ruler was used under a dissecting binocular. The eggs or embryos were then placed in a 10 cc. graduated cylinder, in which they were washed with several volumes of one-tenth Ringer's solution. All excess water was then drawn off, and 2 cc. of clean one-tenth Ringer's solution were added. The volume of eggs plus water was thus determined. Two-tenths of a cubic centimeter of 5 per cent KOH was now added to the side arm of the Warburg or Fenn flask. The eggs and water were then pipetted into the main chamber of the flask. This was now connected with the tap of the manometer or volumeter and placed in the constant-temperature bath. The apparatus was shaken with stopcocks open to the air for 15 minutes, to attain temperature equilibrium. The stopcocks were then closed and the shaking continued. The rate of shaking in most experiments was eighty-five complete oscillations per minute. Until closure of the neural folds the rate of shaking may be decreased to sixty per minute without affecting the rate of uptake. The amplitude of shaking in the Warburg manometer experiments was approximately 9.5 cm.; in the Fenn apparatus it was about 5 cm. The temperature of the water bath was kept constant at 19°8 C. ± 0.01°. One series of experiments was carried out at 14°8 ± 0.02°.

In several series of eggs, along with the rate of oxygen consumption, the wet and dry weights were determined either of the eggs used in the experiment or of eggs from the same batch and at the same stage of development. Wet weight was determined by weighing the eggs or larvae (minus their membranes) on a watch glass or slide, after as much water as possible has been absorbed from the vicinity of the egg by means of filter paper. Great care must be taken to prevent additional loss of water by evaporation. The eggs were then placed in an oven at 110°–120° C. and left overnight. The dry weight was then determined.

RESULTS

Eggs which were removed from the uterus and placed in the respirometers within 40 minutes after fertilization began to consume oxygen immediately. There was no sign of the positive pressure reported by Bialaszewicz and Bledowski, which was attributed by these authors to the evolution of an oxygen-nitrogen gas mixture, resulting from the difference between the pressure of these gases in the body and in the atmosphere.

No detailed comparison of the respiration of unfertilized and fertilized eggs was made, since this question has been fully investigated by previous workers, who are all agreed on the occurrence of a slight increase in rate after fertilization. As the result of experiments on batches of eggs from three different females, it was found that the average oxygen consumption of 100 unfertilized eggs per hour was 5.86 cu. mm., while that of fertilized eggs from the same females was 5.98 cu. mm.

TABLE 1

RATE OF OXYGEN CONSUMPTION STARTING 2.5 HOURS AFTER FERTILIZATION

Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)	Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)
4:30–5:30	4.98	6:00–7:00	5.22
4:45–5:45	5.38	7:00–8:00	6.12
5:00–6:00	5.44	8:00–9:00	5.74
5:15–6:15	5.46	8:30–9:30	5.82
5:30–6:30	5.92		

During cleavage the rate of respiration tends to rise, as can be seen from Table 1 and as has been indicated previously by Brachet (1934). This table shows the observed rates of oxygen consumption, with the Fenn volumeter, per 100 eggs for one batch on which observations started at the beginning of first cleavage. Whether there are fluctuations corresponding to the cleavage rhythm, which have been reported by Brachet (1934) and Stefanelli (1937), could not be determined in the present instance, since the apparatus was not sufficiently sensitive to note such fluctuations. The fluctuations which are seen to occur in Table 1 were noted in later stages and are assumed to be due to experimental errors. Despite the fluctuations the rate of respiration continues to rise, until, before gastrulation, it is double the rate during early cleavage. In Table 2 are shown the observations on the same eggs, started before the beginning of gastrulation and continued until some time after gastrulation had begun. It will be seen from these data that there is no prolonged period over which the rate of oxygen consumption

TABLE 2

RATE OF OXYGEN CONSUMPTION DURING GASTRATION

Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)	Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)
3:00–4:00	11.70	4:02–5:02	12.02
3:17–4:17	11.70	4:17–5:17	12.10
3:30–4:30	11.62	4:32–5:32	12:42
3:45–4:45 (beginning of gastration)	11.94		

remains constant, as Parnas and Krasinska (1921) concluded. Nor does the rate jump abruptly at the beginning of gastrulation, but tends to mount steadily.

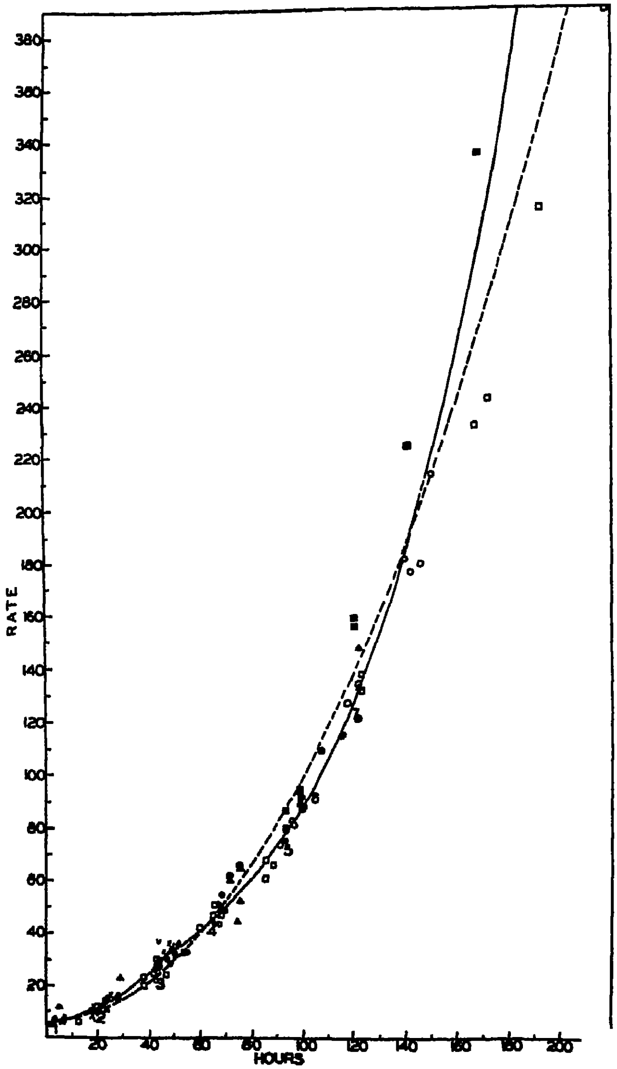


FIG. 1.—Rate of oxygen consumption per 100 eggs per hour during development. The abscissas represent hours after fertilization. The broken curve is for the equation $y = 0.00925t^2$. The full curve from 0 to 47 hours is for the equation $y = 5.31e^{0.038t}$. The full curve after 47 hours has the equation $y = 31e^{0.019(t-47)}$.

A detailed study of the combined data indicates a similar conclusion for the later stages. In Figure 1 are presented the results obtained with the Fenn volumeter. The abscissas indicate hours of development; and the ordinates, rate of oxygen consumption for 100 eggs per hour. Similar symbols are for eggs from the same female. The numerals near the points refer to the following stages of development: (1) first cleavage; (2) beginning of gastrulation; (3) slit blastopore to neural plate; (4) neural folds fusing; (5) hatching (about 4.5 mm. length); (6) heart beat visible (about 5 mm.); and (7) gill branches forming, circulation visible (about 6 mm.). The data obtained with the Warburg apparatus show similar trend but are, on the whole, more variable, and have been omitted in order to prevent confusion. Drawn through the data are two curves. The broken single curve is for the relation $y - a = kt^2$, which was found by Bialaszewicz and Bledowski (1915) to approximate their data (y = rate of oxygen consumption; a = rate of unfertilized eggs; k = constant determined by the data; and t = time). This curve also approximates the present data but is not satisfactory, particularly in the region from 40 to 55 hours, when most of the observed data are above the curve, and in the region from 95 to 120 hours, when most of the observed data are beneath the curve.

The full curve consists of two components. This curve (up to 47 hours) is drawn in an attempt to fit the data with the exponential equation $y = Ae^{kt}$, in which y = rate of oxygen consumption of 100 eggs per hour; A = rate of oxygen consumption at fertilization (a theoretical value obtained in fitting this equation, but approximately equal to the observed value); k = a constant determined by the data; and t = the time in hours after fertilization. To fit the data after 47 hours, the constants must be changed. The values of these constants is indicated in the legends. If this equation represents the correct relations between rate of respiration and time, there must be a slight discontinuity in the orderly increase of rate after the onset of neural-fold formation (after 47 hours). A more marked break was suggested by the data obtained with the Warburg apparatus and an attempt to check this was the original stimulus for the work with the Fenn apparatus.

One difference between the work of Parnas and Krasinska and that of others is the fact that the former workers made their observations at a lower temperature: they worked at 15° C. For this reason one series of data was collected as follows: The oxygen uptake was noted at a temperature of 14°.8 for 1–2 hours. The stopcocks of the manometers were then opened, and the temperature of the bath was changed to 19°.8. The manometer vessels were equilibrated for 1 hour at this temperature, and the stopcocks were then closed. Oxygen consumption was then determined at this second temperature on the same eggs. In this way it was possible to study the effect of temperature on the same material at approximately the same stage of development.

In Figure 2 the results of this parallel run are given. The curves for the 20° series are for the same exponential equations as those in Figure 1. The curves for the 15° series merely follow the data. No equation was determined for them. The dotted curve is one derived from the 15° data and represents the probable rate of increase of respiration of a batch which would develop at 15° throughout. Although the data are not sufficient to exclude the possibility of abrupt changes at critical stages, still their general course indicates the likelihood of a gradual increase in rate at this temperature also. These data further tend to corroborate the possibility of a slight discontinuity in the neighborhood of 50 hours. The temperature coefficient (Q_5) is approximately 1.8 for each stage. There is, however, insufficient data to determine whether there are any significant changes during development. A value for the Q_5 for development over this period can be obtained by interpolation in Figure 5 of the paper of Atlas (1935). This indicates a value of 1.88 for the Q_5 of development. Within the limits of experimental error these are sufficiently close to indicate that the temperature coefficients of these simultaneous events are close together, if not identical. Although the temperature coefficients are higher than those of Tyler (1936) for various invertebrate eggs, they agree with his results in that the coefficients are practically identical for rate of development and for rate of oxygen consumption.

It was also considered desirable to obtain data from *R. sylvatica*. The eggs of this species are laid earlier, and generally at a lower temperature than those of *R. pipiens*. In this regard and in their heat tolerance they resemble *R. fusca* (both develop normally between 2° and 22°5, whereas *R. pipiens* has a normal development range between 6° and 30°). Experiments were carried out on the eggs of *R. sylvatica*, which were collected in ponds near New York in March and April, 1937. The details of the experiments were identical with those given for *R. pipiens*; and the general conclusions are similar, in that no indications of abrupt changes appear. The data are plotted in Figure 3, in which are included the points (*B*) which represent the data for *R. fusca* (Bialaszewicz and Bledowski, 1915, Fig. 4, p. 462) and the curve for *R. pipiens* (this paper, Fig. 1) for comparative purposes. The *R. sylvatica* data approximate those for *R. fusca*. The rate for each of these species is higher than that observed for *R. pipiens* at identical hours after fertilization. The

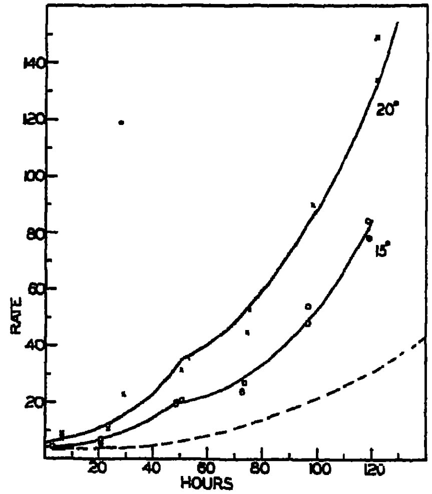


FIG. 2

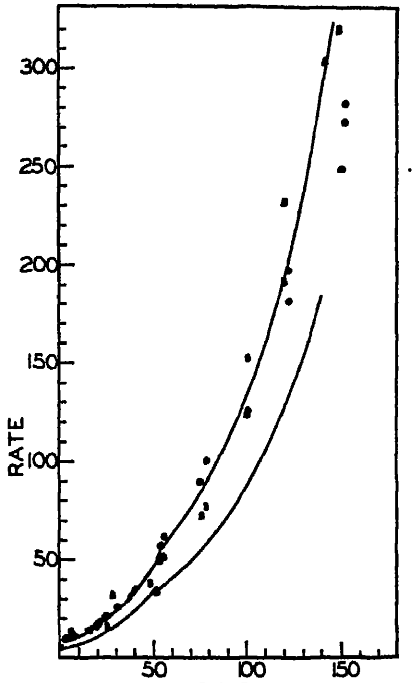


FIG. 3

FIG. 2.—Rate of oxygen consumption per 100 eggs per hour during development at 20° and 15° C. The broken curve is a calculated curve showing the rates of oxygen consumption related to time taken to develop at 15° C.

FIG. 3.—Rate of oxygen consumption per 100 eggs per hour during development of three species of frogs. The black circles are for *R. sylvatica*. The symbol *B* is for data from *R. temporaria* (*fusca*), from Bialaszewicz and Bledowski (1915). The lower curves are for *R. pipiens* from Fig. 1. The upper curves are $y = 7.5e^{0.038t}$ up to 53.3 hours and $y = 56e^{0.019(t-53.3)}$ after 53.3 hours.

dotted curves are fit to the equation $y = Ae^{kt}$, in which the terms have the same meaning as above (Fig. 1); and again there is an indication of a slight discontinuity in the neighborhood of 50 hours.

Data on the growth of these embryos are presented in Figure 4. The abscissas represent hours of development. The upper ordinates represent millimeters (longest diameter up to 50 hours, length after 50 hours); the lower ordinates represent milligrams wet weight. It will be seen that the length dimension increases slowly, and approximately linearly from 1.5 mm. at zero hours of development to 1.8 mm. at 50 hours (which is at the middle of the neural folds stage). Starting with this stage, the rate of increase of length becomes rapid; but at about 120 hours (when gill circulation has recently begun) the rate of growth of length decreases. The *R. sylvatica* data (indicated by the letter *S*) follow the same general course except that they are slightly in advance at each time-interval. The curve is for the smoothed data of *R. pipiens*.

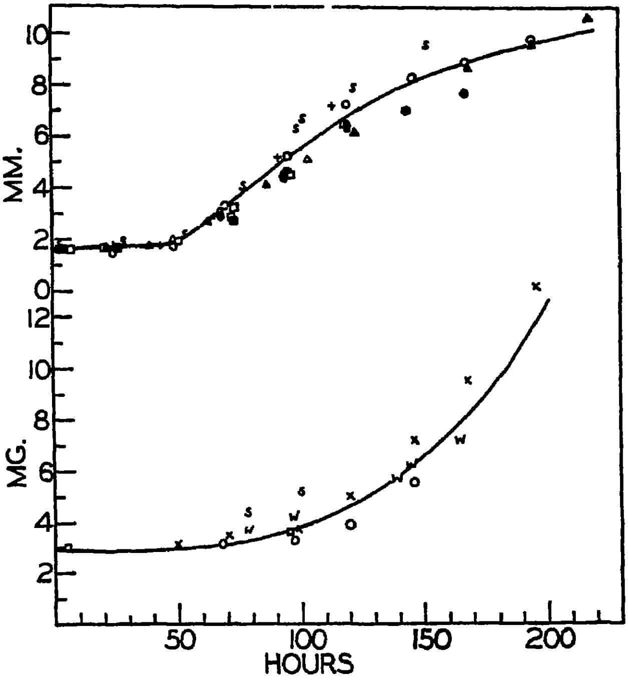


FIG. 4.—Growth of embryos and larvae during development. Similar symbols indicate data from the same batch of eggs. *S* represents data for *R. sylvatica; W* represents data for *R. sylvatica* from Williams (1900), obtained in the present form by interpolating from Fig. 6 of this paper into the upper curve of this figure. All other points are for *R. pipiens*. MM. = length in millimeters; MG. = milligrams wet weight.

In the lower curve of Figure 4 is represented the change in wet weight during development. The wet-weight data indicate that there is only a slight uptake of water before 50 hours, with a more rapid increase thereafter. Represented are the observed points from two batches of eggs. The curve drawn represents the average values obtained from many other batches of eggs. Also included are original observations (*S*) made for *R.*

sylvatica and the data (*W*) of Williams (1900); also for *R. sylvatica* (obtained by interpolation from Fig. 6, this paper). The data of Williams are of interest since they indicate that, although the initial wet weight is higher for *R. sylvatica* than for *R. pipiens*, the relation is reversed later, apparently because of the more rapid intake of water by the latter species. If we compare the two curves of this figure, it becomes apparent that increase in length precedes increase in wet weight and follows an entirely different course.

DISCUSSION

Earlier work on the development of various eggs has indicated a rise in the rate of oxygen consumption in most cases. Although there are many gaps in previous data, the evidence in general tends to indicate that there is a gradual increase in rate of respiration during development, without any abrupt changes. This is demonstrated by data for echinoderms by Warburg (1915), Meyerhof (1911), Rapkine (1927), and Ephrussi (1933); for the bryozoan *Aplysia* by Buglia (1908) and Meyerhof (1911); for insects by Fink (1925); for the chick by Bohr and Hasselbach (1900) and Murray (1925); for gastropods by Baldwin (1935); for annelid eggs by Whitaker (1933).

The earlier work on amphibian eggs also supported a continuous increase (Godlewski, 1900; Bialaszewicz and Bledowski, 1915). However, the work of Parnas and Krasinska (1921) tended to indicate periods during which the respiration remains constant, followed by abrupt changes in rate. Parnas and Krasinska concluded that the rate rose abruptly at the beginning of gastrulation, neurulation, and external gill growth, with no noticeable changes in between. Hyman (1921) has presented data for *Fundulus* which indicate a decline in rate after gastrulation.

In the present paper an attempt has been made to reinvestigate this question for amphibian eggs. As can be seen from the data in Tables 1 and 2 and Figures 1, 2, and 3, there is no indication of abrupt changes. The evidence seems to indicate a gradual rise. An examination of the data of Parnas and Krasinska leads to some clues as to the deviation of their views from those of this paper. Probably the greatest factor, indicated by their data, which contributes to their conclusions arises from the measurement of *total* oxygen uptake from the beginning of the experiment (which lasted over 4 days in some cases). As a result their data concerning rate of oxygen consumption are obtained indirectly from the total oxygen consumed. Such a procedure might be countenanced if the observations were sufficiently close to permit the drawing of an accurate curve. But there are large gaps in the data in many cases. This, together with the fact that the temperature at which the experiment was conducted showed fluctuations as much as 2° in some cases, might account for the particular conclusions that these workers reached.

The gradual increase in the rate of respiration is in harmony with observations of embryologists which indicate a continuous increase in cell numbers during the period of development that has been studied in this paper. The increase in rate of respiration of the embryo, taken as a unit, reflects the growth of cell population according to the view of the present author. The question arises whether there is any simple relation which can describe the changing rate of respiration. As indicated in Figure 1, the data which have been obtained can be described by the equation $y = 5.31e^{0.038t}$ from 0 to 47 hours of development, in which the terms have the meaning defined above. The chief value of the use of this equation is that it offers the working hypothesis that during early development the respiratory mechanism is being built up at a uniform rate, and that the quantity of respiratory material present at any instant is greater by a constant percentage than the amount present during the immediately preceding instant.

After 47 hours there is a change in the value of the constants of the foregoing equation, as indicated in Figure 1. This change may be correlated with other changes which are occurring in the embryo at this stage, which corresponds to the onset of neural-fold formation. One of these changes is the increase in length which begins to occur about this time, as shown in Figure 4. The relation between rate of respiration and length is brought out in Figure 5. Here, on the lower abscissa, is plotted length in millimeters; and on the ordinate scale, rate of respiration. Length refers to total body length from tip of head to tip of tail; however, before the body axis is formed, length refers to the diameter of the egg. The plot shows that the rate of respiration increases rapidly, relative to length during early development. However, when length begins to increase more rapidly (at about 50 hours, or 2.1 mm. in Fig. 4) the rate of respiration follows the increase of length in accordance with the equation $y = 40e^{0.28(L-2.1)}$. Here y = rate of respiration of 100 eggs per hour, e = the base of natural logarithms, L = length in millimeters. The fit of this equation permits the hypothesis that after neural-fold formation the increase in rate of respiration becomes a constant percentage of the increase in length. Since the increase in length of these embryos is due largely to growth of the tail (Atlas, 1935, p. 304) and since the growth of the tail is due largely to an increase in cell number by cell division, the implication is that there is a constant percentage increase in cell number when the length of the embryo increases, and a resultant constant percentage increment in rate of respiration. According to this interpretation, the growth of the respiratory function is probably due mainly to an increase in total number of cells rather than to an increase in intensity of respiration of individual cells.

During the early growth of the frog larvae, one of the outstanding changes is the increase in water content, as shown in Figure 4. It is interesting to note how the rate of respiration behaves with respect to this variable. Therefore, in Figure 5 there is also plotted the rate of respiration with increase in wet weight (expressed in milligrams on the upper abscissa). It will be seen that the rate of respiration increases more rapidly relative to wet weight at first, that then the rate of increase slows up, and that during later stages the rate of respiration tends to approach an asymptote. This would tend to indicate that the rate of respiration is not increasing proportionately to increase of the volume of the embryo and that much of the water which is entering is not made part of the respiratory mechanism.[1] It must not be inferred from this, however, that the water is actually impeding the increase in the amount of respiratory mechanism. This follows from a comparison of Figures 5 and 6. In Figure 6 is plotted the relation between wet weight and length for *R. pipiens*, *R. sylvatica*, and *R. fusca*. The letters indicate the authorities from which the data were derived. It will be observed that, after 5 mm., the larvae of *R. pipiens* have more water, at equal lengths, than do those of *R. sylvatica*. In Figure 5 is plotted the rate of respiration of larvae of *R. sylvatica* at different lengths. Although the equation is slightly different for *R. sylvatica* than for *R. pipiens*, we can observe that for equal lengths of larvae the rate of respiration is approximately equal for the two species (within the limits of experimental error). In other words, despite the greater amount of water for equal lengths which the larvae of *R. pipiens* have, they respire at the same rate as those of *R. sylvatica*.

---

[1] In Figure 6 it is shown that the change due to dry weight is negligible and may be omitted from this discussion. The change in wet weight is therefore a measure of the increase in water content.

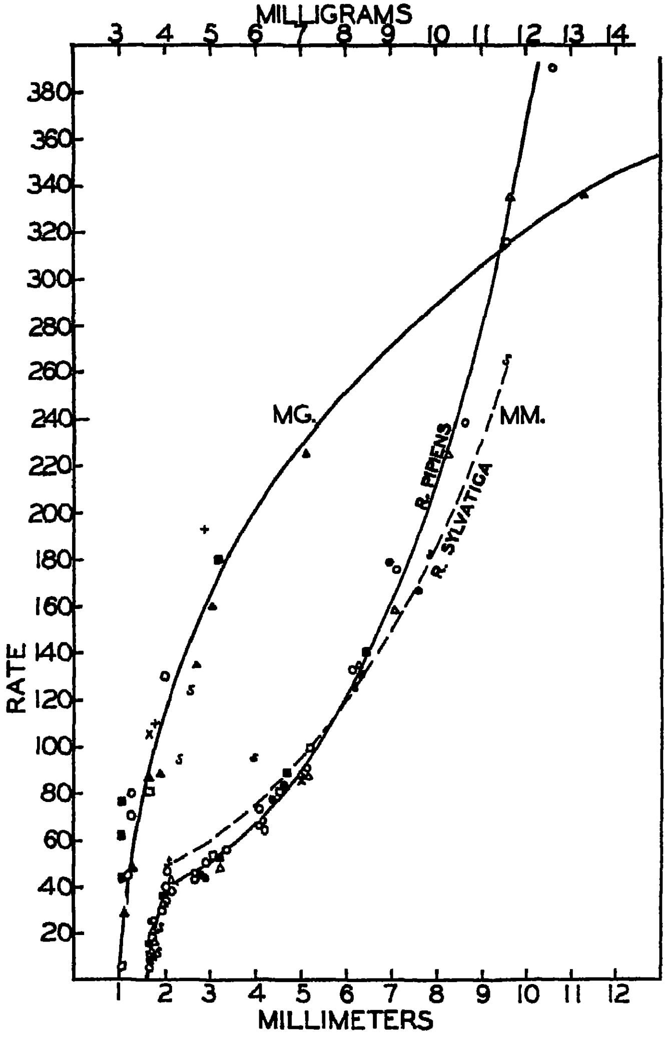


FIG. 5.—Relation between rate of oxygen consumption per egg per hour and length and wet weight. The symbol *S* and broken curve are for *R. sylvatica*. The *R. pipiens* rate-length curve is for the equation $y = 40e^{0.28(L-2.1)}$. The *R. sylvatica* curve is for $y = 50e^{0.22(L-2.1)}$.

Another matter of interest in connection with Figure 6 is that the points of *R. sylvatica* and *R. fusca* fall on approximately the same curve. This may be of significance in connection with the data presented in Figure 3 in which one can see that the rates of respiration at equal times after fertilization also correspond for these species. The two sets of data, however, cannot be further analyzed, since there are no observations at hand which indicate the rate of length increase of *R. fusca* at a temperature of 20° C. For this reason a study of respiration at different lengths is not possible for *R. fusca*.

Also plotted in Figure 6 are the changes in dry weight which have been observed for *R. pipiens* (*x*) and for *R. sylvatica* (*w*) (data of Williams). The lines drawn connect observations made on eggs of *R. pipiens* taken from the same females. The two outer lines represent the maximum and minimum weights observed, while the middle line is from an intermediate batch of eggs. As has been noted by previous authors, the dry weight of the frog's egg diminishes during development (in the present case from a mean of 1.3 mg. at the beginning of development to one of 1.1 mg. when the external gills are covered or a difference of 0.2 mg.). It will be seen that the total loss throughout development is not appreciably larger for the eggs with more initial dry matter than for those with less. The total amount lost seems to be proportional to the length rather than to the initial dry weight.

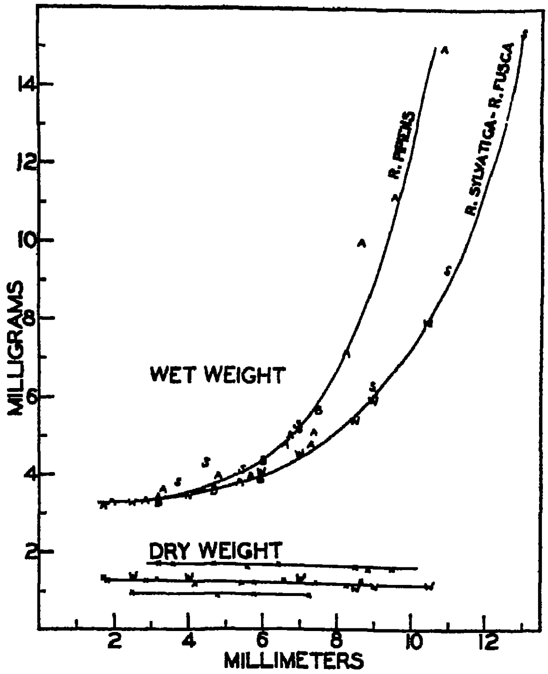


FIG. 6.—Relation of wet and dry weight to length in millimeters. Upper curve for wet weight; lower curve for dry weight. *A*, data for *R. pipiens* this paper; *B*, data for *R. temporaria* (Bialaszewicz, 1908); *S*, data for *R. fusca* (Schaper, 1902); *W*, data for *R. sylvatica* (Williams, 1900).

By graphical integration of the full curves of Figure 1 the total amount of oxygen consumed during the first 200 hours of development at 20° C. can be calculated. This is approximately 250 cu. mm. for each embryo. The amount of oxygen necessary to oxidize 0.2 mg. of the dry weight of the embryo would be 149, 191, or 400 cu. mm., if glucose, protein, or fat, respectively, were the primary material being oxidized (Hawk and Bergeim, 1937). From these figures it is likely that a certain amount of fat is oxidized during the period studied. However, since the respiratory quotient is approximately 1 from gastrulation to hatching (Brachet, 1934), very little fat is burned during this period. The greater amount of fat must therefore be oxidized between hatching and the covering of the external gills. This is an indirect confirmation of the work of Bialaszewicz and Mincovna (1921) on *R. fusca*, who found that the disappearance of fat does not begin until after hatching of the frog larvae. This also implies that the respiratory quotient probably falls after hatching.

SUMMARY

1. The rate of oxygen consumption increases gradually, without sudden jumps, during the development of *R. pipiens* and *R. sylvatica* from fertilization to overgrowth of the operculum (Figs. 1 and 3).
2. The relation between the rate of oxygen consumption per hour per embryo and time may be expressed by the exponential equation $y = Ae^{kt}$, providing a change of constants is introduced for data after the neural-fold stage.
3. After the neural-fold stage the relation between rate of oxygen consumption (y) and length (l) of embryo may be expressed by the equation $y = Ae^{KL}$ (Fig. 5).
4. The temperature coefficient (Q_5) is approximately the same (1.8) for rate of development and rate of oxygen consumption during development of *R. pipiens* at 15° C. and 20° C. (Fig. 2).
5. Increase in length of embryo precedes the rapid increase in water intake (Fig. 4).
6. At equal lengths *R. pipiens* and *R. sylvatica* respire at the same rate (Fig. 5) even though the *R. pipiens* larvae have more water.
7. The increase in rate of respiration per embryo is a function of the cell number rather than of the intensity of respiration of individual cells.
8. The total oxygen consumed during the first 200 hours of development of *R. pipiens* at 20° C. is approximately 250 cu. mm. The total decrease in dry weight is approximately 0.2 mg. From this it is deduced that there is probably a combustion of fat after hatching.

The author wishes to acknowledge his indebtedness to the department of zoölogy of Columbia University for facilities to carry on this work, and to Professor L. G. Barth, who helped in its progress. Some of the observations were made with the aid of Mr. N. Stevens.

LITERATURE CITED

ATLAS, M. 1935. The effect of temperature on the development of *Rana pipiens*. Physiol. Zoöl., 8:290–310.

BALDWIN, ERNEST. 1935. The respiratory quotient of developing gastropod eggs. Jour. Exper. Biol., 12:27–35.

BATAILLON, E. 1896. Nouvelles recherches sur les mécanismes de l'évolution. Les premiers stades du développement chez les poissons et les amphibiens. Arch. zool. expér. et générale, 5:281–317.

———. 1912. La Parthénogénèse des amphibiens et la "fécondation chimique" de Loeb. Étude analytique. Ann. sci. nat., n.s., 16:249–307.

BIALASZEWICZ, K. 1908. Beiträge zur Kenntnis der Wachstumsvorgänge bei Amphibionembryonen. Bull. internat. Acad. sci., Cracovie. Cl. des sci. math. et nat., pp. 783–835.

BIALASZEWICZ, K., and BLEDOWSKI, R. 1915. The influence of fertilization on the respiration of eggs. Pro. Sci. Soc. Warsaw, 8:429–73.

BIALASZEWICZ, K., and MINCOVNA, M. 1921. Sur le métabolisme des principes gras et azotés aux premiers stades du développement de la grenouille. Trav. du Lab. de physiol. de l'Institut Nencki, 1:1–23.

BOHR, CHR., and HASSELBACH, K. 1900. Ueber die Kohlensäureproduction des Huhnerembryos. Skandinav. arch. f. physiol., 10:149–73.

BRACHET, J. 1934. 1. La Respiration et la glycolyse de la segmentation à l'éclosion (*Rana fusca*). Arch. de biol., 45:611–727.

———. 1935. 2. La Respiration de l'œuf pendant la fécondation et la mitose (*Rana fusca*). *Ibid.*, 46:1–24.

BUGLIA, G. 1908. Sullo scambio gassoso delle uova di *Aplysia limacina* nei vari periodi dello siviluppo. Arch. di fisiol. 5:455–70.

DAVENPORT, C. B. 1897. The role of water in growth. Proc. Boston Soc. Nat. Hist., 28:73–84.

DIXON, M. 1934. Manometric methods. Cambridge University Press.

EPHRUSSI, B. 1933. Contribution à l'analyse des premiers stades du développement de l'œuf. Action de la température. Arch. de biol., 44:1–147.

FENN, WALLACE O. 1927. The gas exchange of nerve during stimulation Amer. Jour. Physiol., 80:327–46.

FINK, DAVID E. 1925. Metabolism during embryonic and metamorphic development of insects. Jour. Gen. Physiol., 7:527–43.

GODLEWSKI, E. 1900. Ueber die Einwirkung des Sauerstoffs auf Entwicklung und uber den Gaswechsel in den ersten Entwicklungstadien von *Rana temporaria*. Bull. Internat. de l'Acad. Sci., Cracovie, pp. 232–55.

HAWK, P. B., and BERGEIM, OLAF. 1937. Practical physiological chemistry (11th ed.). P. Blakiston's Son & Co. Philadelphia.

HYMAN, LIBBIE H. 1921. The metabolic gradients of vertebrate embryos. I. Teleost embryos. Biol. Bull., 40:32–73.

MEYERHOF, OTTO. 1911. Untersuchungen über die Warmetönung der vitalen Oxydationsvorgänge in Eiren. Biochem. Zeitschr., 53:280–328.

MURRAY, H. A. 1925. Catabolism. Chemical changes in fertile eggs during incubation. Selection of standard conditions. Jour. Gen. Physiol., 9:1–37.

PARNAS, J. K., and KRASINSKA, S. 1921. Über den Stoffwechsel der Amphibienlarven. Biochem. Zeitschr., 116:108–37.

RAPKINE, LOUIS. 1927. Les Echanges gazeux et la nature des oxydations chez l'œuf fécondé d'oursin (*Paracentrotus lividus*). Compt. rend. Soc. biol., 97:143–49.

RUGH, R. 1937. Ovulation induced out of season. Science, 85:588–89.

SCHAPER, A. 1902. Beiträge zur Analyse des tierischen Wachstums. Eine kritische und experimentelle Studie. Arch. f. Entw'mech., 14:307–401.

STEFANELLI, ALBERTO. 1937. A new form of micro-respirometer with a note on the effect of cleavage on the respiration of the egg of *Rana*. Jour. Exper. Biol., 14:171–78.

TYLER, A. 1936. Comparison of the rates of oxygen consumption and development at different temperatures of eggs of some marine animals. Biol. Bull., 71:82–100.

WARBURG, O. 1908. Beobachtungen über die Oxydationsprozesse im Seeiglei. Zeitschr. f. physiol. Chem., 57:1–16.

———. 1915. Notizen zur Entwicklungsphysiologie des Seeigeleis. Pflügers Arch., 160:324–32.

WHITAKER, D. M. 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. *Chaetopterus* and *Arbacia punctulata*. Jour. Gen. Physiol., 16:475–95.

WILLIAMS, S. R. 1900. The specific gravity of some fresh water animals in relation to their habits, development and composition. Amer. Nat., 34:95.

WILLS, IRWIN A. 1936. The respiratory rate of developing amphibia with special reference to sex differentiation. Jour. Exper. Zoöl., 73:481–510.

LIGHT INTENSITY AND MELANOPHORE RESPONSE IN A CYPRINID FISH[1]

RAGNAR NATHANAEL DANIELSON
University of Minnesota

THE pigmentary responses of fishes which show adjustment to background are known to be in accordance with the degree of contrast between the background and the rest of the environment under all ordinary circumstances. The intensity of illumination appears to be relatively unimportant in these responses. Such men as Sumner (1911), Mast (1914), Murisier (1921), Koller (1934), and others have concluded, in general, that the shade assumed by normal fishes is almost independent of the intensity of the light to which they are exposed. While Duspiva (1931) found marked differences in the shade of embryonic and early larval individuals of certain species, these fishes showed no clear response to background, and thus formed no exception to the general rule.

The question naturally arises whether or not the rate of color change differs at different intensities of illumination, even though the amount of change may be approximately constant. When the experiments to be described were undertaken, however, no accounts of quantitative work dealing with this point could be found in the literature. It seemed desirable, therefore, to attempt to determine the time required for melanophore change at each of several different light intensities.

The river chub, *Nocomis biguttatus* Kirtland, was chosen as the experimental animal, chiefly because of its convenient size and striking melanophore change. The individuals used, all of which were obtained from a Minneapolis bait-dealer, ranged in length from about 11 to 15 cm. When kept for any considerable period, they were occasionally fed chopped liver, earthworms, or very small minnows.

All trials were made in a cylinder consisting of a wooden frame, 40 cm. in diameter, covered with heavy, light-proof, black paper. The top of this cylinder was covered by one or more sheets of opal glass; and when still greater reduction of light intensity was necessary, sheets of pure white bond paper were inserted between the glass plates. The source of light was a Mazda lamp supported directly above the center of the cylinder by a crossarm attached to an upright pipe standard having a height of about 1½ meters above the cylinder. The crossarm could be clamped at any desired distance above the cylinder, up to the end of the pipe. In this way, exact adjustments of the light intensity within the cylinder were possible by varying the number of sheets of opal glass and paper and the distance of the lamp above them. Two clear Mazda lamps were used—1,500 watts for the highest intensity, 300 watts for all others.

The fish to be tested were placed in a white enamel or dead-black glass dish about 20 cm. in diameter and 8 cm. deep, containing 4 cm. of water. This depth of water was about the minimum in which the fish would remain reasonably quiet under the experimental conditions. The surface of the water was 25 cm. from the lower side of the diffusing opal glass. Openings made in the side of the cylinder were covered with several layers of black cloth in such a way that both hands could be introduced to manipulate the dishes containing the fish without admitting additional light.

---

[1] I am indebted to Dr. D. E. Minnich, chairman of the department of zoölogy of the University of Minnesota, for his aid in securing materials and facilities for this investigation, as well as for criticisms both of the experimental work and the interpretation of its results.

The intensities used were 100, 10, 1, 0.1, and 0.01 foot-candles as determined by the use of a Weston photronic illuminometer at a position corresponding to the surface of the water.[2] The actual intensity reaching the fish in the water was, of course, somewhat less. Water temperatures were kept as constant as possible, but varied between 14° and 19° C. during most of the work. Part of the time a room was available in which the temperature varied only between 15° and 18°, and the fish were then kept in this room at all times.

The relatively crude method of direct observation of individual fish was used. The time was taken from the moment when the fish were placed upon the background—black or white, as the case might be—until it was judged that change was complete. Obviously, this method offers too great possibilities of error for very exact results; but because of the scarcity of material and for various other reasons, it seemed to be the only feasible method.

No standard of comparison could be devised which proved any more satisfactory than the subjective judgment of the individual fish. It was found with practice, however, that results could be obtained with a given individual under a given set of conditions which agreed surprisingly well. It is probable, to be sure, that the absolute end-point was frequently not obtained; but the remaining change which might have taken place in any particular fish was, at least, nearly negligible as far as general appearance was concerned. The results, therefore, appear accurate enough for comparative purposes.

Observations were made by quickly removing the filters covering the top of the cylinder, and at the same time changing a double-throw switch, which cut off the original light and substituted the light from another 300-watt lamp at a fixed distance. Thus, observations were always made at the same illumination. Because of the very rapid color change of this minnow, the condition of the fish then had to be judged within a few seconds as either completely or incompletely changed. Later tests were then made for a shorter or longer time, depending on whether change was complete or not, until the time limits within which the particular fish would and would not undergo complete change had been determined.

It was found necessary to put each fish through a period of preliminary "practice" lasting for a week or two before using it for the tests. As noted by Pouchet (1876) and many later investigators, the time required by fishes for color change can often be considerably reduced by such practice, and it was found that *Nocomis* in this respect formed no exception to the rule. Unless the fish were first put through this practice period, the time required for color change would continue to decrease for a considerable time. The practice period also served several other purposes. It allowed the experimenter to become familiar with the appearance of the individuals, and thus promoted more accurate judgment as to the completion of melanophore change. It made the fish much more tame and easy to handle in critical situations. Finally, it made the appearance of the fish more constant, for all fish lost more or less pigment under laboratory conditions, and most of this loss occurred during the preliminary period. One of the unavoidable results of the necessity of keeping the fish for such a long time, however, was that all experiments were performed with individuals which were relatively pale and poor in pigment.

---

[2] The lowest intensity used may not have been of the exact value given, since it was near the limit of sensitivity of the measuring instrument used, and reading it required estimation of half a scale division. Numerous checks in which the number of filtering sheets and the lamp distance were varied without reference to previous settings seemed to indicate, however, that, whatever its absolute value, this intensity was at least as constant as any of the others.

In the first series of experiments the fish were first placed for 1 hour on a white background under an illumination of 10 foot-candles and then transferred to black background at the test intensity. Observations were then made, as already described. The results obtained with 11 individuals are shown in the columns headed "A" in Table 1. The figures represent the limits of time in minutes within which change was judged to have been completed. From three to eight or ten readings were made to establish each limit, depending on the difficulties of judgment in the individual case.

TABLE 1

TIME (IN MINUTES) REQUIRED FOR CHANGE FROM LIGHT TO DARK CONDITION

A = after adaptation for 1 hour on white background at illumination of 10 foot-candles; B = after adaptation for 1 hour on white background at same illumination as test.

Fish No.	A 100 Ft.-C.	A 10 Ft.-C.	A 1 Ft.-C.	A 0.1 Ft.-C.	B 0.1 Ft.-C.	A 0.01 Ft -C.	B 0.01 Ft.-C.
1	9–10	8– 9	8–10	18–20	30–35?	Inc. 120	Inc. 120
2	8–10	8–10	8– 9	12–15	8–10	25–30	9–10
3	8–10	8–10	8–10	15–18	8–10	25–30	8–10
4	10–15	9–10	8–12	18–20	14–15	30–35	9–10
7	14–15	14–15	16–18	Inc. 120*	Inc. 120	Inc. 120	Inc. 120
11	9–14	9–10	10–14	Inc. 120	Inc. 120	Inc. 120	Inc. 120
13	4– 6	3– 4	4– 5	Inc. 120	Inc. 120	Inc. 120	Inc. 120
14	4– 6	4– 6	4– 6	Inc. 120	Inc. 120	Inc. 120	Inc. 120
22	8–12	10–12	9–10	12–15	10–12	25–30	18–20
23	4– 6	3– 4	4– 6	18–20	6– 8	25–30	4– 5
24	4– 6	4– 6	4– 5	18–25	3– 4	18–20	3– 4
Av.	7.5–10	7.3– 8.7	7.5– 9.5	16–19	8.1– 9.8	25–29	8.5– 9.8

* Inc. 120 = change definitely incomplete after 120 minutes.

The average time required for change by the 11 fish was between 7 and 10 minutes at all three of the higher intensities—100, 10, and 1 foot-candles. A definite increase in the time required for change was seen at 0.1 foot-candle, and a further increase at 0.01 foot-candle. It is important to note that 4 fish definitely failed to turn completely dark even in 2 hours at 0.1 foot-candle while 5 failed to do so at 0.01 foot-candle. The significance of this will be discussed later. In the light of later results and in view of the work of Brown (1936), it now seems certain that the others were also incompletely dark at these intensities; but at least the condition of their melanophores was sufficiently close to complete expansion to be nearly indistinguishable by gross observation in the absence of a satisfactory standard of comparison.

In attempting to discover what was involved in the increase of reaction time at low intensity, a second series of readings at the two lower intensities was made. In this case the fish were first adapted for 1 hour on a white background at the same intensity for which their adaptation time on black was determined. The results are shown in the two columns marked "B" in Table 1. The fish which had not turned completely dark under the previous experimental conditions still did not do so. The lengthening of adaption time previously shown by the others at intensities below 1 foot-candle was, however, definitely reduced. Fish No. 1 was an apparent exception, having required an even longer time to change than before; but since it died shortly afterward, this difference was probably due to some change in its physiological condition.

These results thus show that, when the fish were preadapted on a white background at 10 foot-candles, the time required for change to the dark condition was approximately constant at intensities of 1 foot-candle or more. At lower intensities, two changes in response appeared: first, a lengthening of adaptation time; and second, a tendency toward incomplete change. With preadaptation at the test intensity, the former tended to be reduced, the latter was unchanged.

TABLE 2

TIME (IN MINUTES) REQUIRED FOR CHANGE FROM DARK TO LIGHT CONDITION

A = after adaptation for 1 hour on black background at an illumination of 10 foot-candles; B = after adaptation for 1 hour on black background at the same illumination as the test.

Fish No.	A 100 Ft.-C.	A 10 Ft.-C.	A 1 Ft.-C.	A 0.1 Ft.-C.	B 0.1 Ft.-C.	A 0.01 Ft.-C.	B 0.01 Ft.-C.
27	1½-2	1½-2	1 -1½	2 -3	1¼-1¾	6 - 7	1 -1¾
28	1½-1¾	1¼-1¾	1 -2	2 -3	1¼-1¾	7 - 8	1 -2
37	1 -1½	1 -1½	1 -1½	1 -1½	1 -1½	7 -11	1 -1½
38	1 -1½	1 -1½	1 -1½	2 -3	1 -1½	4 - 6	1 -1½
26	1¾-2¼	1½-2	1½-2	1½-2	....	1¼- 1¾	1½-2
36	....	1 -1½	....	1 -1½	....	1 - 1½	¾-1¼
40	....	2 -2½	....	....	....	3 - 4	1½-2½
44	....	1 -1½	....	....	....	4 - 5	1 -1½
45	....	1 -1½	....	....	....	5 - 6	1 -1½
48	....	1 -1½	....	....	....	8 -10	1 -1½
Av.	1.4-1.8	1.2-1.7	1.1-1.7	1.6-2.3	1.1-1 6	4.6- 6 0	1.1-1.7

For a complete study of the problem, it seemed desirable to study adaptation in the reverse direction, namely, from dark to pale condition. It was impossible to complete this study with the same animals, but the results of a corresponding series obtained with other individuals appear in Table 2. Because of deaths, only 4 of the 10 fish represented could be followed through completely at all intensities; but these, as well as the other 6 on which determinations were completed at a few intensities, show results similar to those for the change from white to black. The lengthening of adaptation time at low intensities seemed to disappear in all cases where preparatory adaptation was made at the test intensity. The principal differences from the previous series of experiments were, first, that no animals definitely failed to undergo complete change at low intensities, as far as could be determined under the conditions of the experiment, and, second, that the time required for change was much shorter.

Thus, on a given background, either white or black, the rate of melanophore change was approximately constant at all of the higher intensities. The time required for change definitely increased at low intensity, however, unless previous adaptation had occurred at the same low intensity. The fact that this increase in the time required for change apparently tended to disappear when the fish were exposed to the same low intensity throughout the experiment might suggest that the factor responsible for the longer adaptation time originally observed was the time required for the adaptation of the eyes to the low illumination. But while this factor may be important, it cannot explain the fact that such a large proportion of the fish definitely failed to turn completely dark at low illumination under any conditions.

A possible clue as to what occurred was offered by the observation that at low intensities, on both white and black backgrounds, it seemed much more difficult than at higher intensities to determine whether complete change had taken place. From this, together with the observed definite failure of a large proportion of the fish to turn completely dark under low illumination, the suspicion arose that the condition on either white or black background under low illumination was an equilibrium short of complete contraction or expansion of the melanophores.

Direct evidence for incomplete change at low intensities was obtained by adapting 5 normal minnows each on white and black background at 0.01 foot-candle for 1 hour, then throwing them into hot formalin solution and comparing pieces of the integument with similar pieces from minnows which had been on similar backgrounds at an illumination of 10 foot-candles for the same length of time. Because of the small number of fish available and the great variation in the size and form of the melanophores, measurement of the melanin masses or other attempts to make strictly quantitative comparisons seemed unlikely to offer any advantage. Conclusions were, therefore, reached by comparing two pieces of integument during several minutes of study and recording the general impression obtained. The conclusions reached were checked in each case by persons who knew nothing of the source of the pieces or the nature of the experiment but were simply asked to decide whether or not there was greater pigment dispersion in one case than another. In no case was there any doubt that under low illumination greater dispersion had occurred on a white background, and less dispersion on a black one, than in the controls.

Thus, neither complete dispersion nor complete contraction of the pigment occurs at low light intensity. It follows, therefore, that under such low intensity the observed time for color change is the time required for change from a partly dark to a partly light condition or the reverse. Consequently, even though the observed adaptation time be as short under some conditions as at higher intensity, the rate of change must have decreased because of the corresponding decrease in the amount of change.

In this connection the response of the fish to complete darkness is also of interest. Careful observations at various times on some thirty specimens of *Nocomis* have shown that in the dark a clearly intermediate condition is gradually reached, regardless of whether the fish are dark or pale to begin with. While exact determinations were not made, the minimum time for a reasonably complete approach to the equilibrium condition in darkness seemed to be about ½–1 hour. The condition, once attained, persisted for at least 24 hours, the longest time the fish were left in the dark, so that this response to darkness is not a transient phenomenon. Various observations on other minnows, such as *Semotilus atromaculatus* and *Pimephales promelas*, have given similar results.

It appears, therefore, that the melanophore change at the lower intensities of illumination approaches the change which occurs in darkness in both rate and amount. In the dark the melanophores slowly reach an intermediate condition of expansion. Under low illumination the time required for change, while less than that required in the dark, is greater than that required in stronger light. Likewise, though the response to background is still dominant even at the lowest intensity used, the expansion of the melanophores is less on black, and greater on white, background than it is at higher intensities.

The photic processes involved in melanophore changes at low light intensity must be limited by the sensitivity of the receptive apparatus. It is interesting to note that Brown (1936), in experiments published shortly after the completion of the foregoing, found 1.75 foot-candles to be the lower limit at which complete melanophore expansion would occur in *Ericymba buccata*, and that below this point expansion was proportional to the intensity. In my own experiments, approximately complete melanophore change at a constant rate appeared to occur at and above about 1 foot-candle. Thus, changes appear to be independent of intensity down to about the same point in both of these minnows. It seems conceivable that the point where melanophore activity becomes dependent on light intensity may be the point where cone vision ceases. It must be borne in mind, however, that fish without eyes, though they no longer respond to background, may still show melanophore responses to changes in illumination, so that the problem of the exact structures and processes involved in reception is not a simple one.

To what extent receptors other than the eyes were involved in the present results is not certain. The effect of light as such is always an expansion of the melanophores. It would appear, therefore, that a fish on either black or white background should be slightly darker if the direct response to light were involved than would otherwise be the case, since the effect of a black background would be reinforced while that of a white background would be opposed. Whether this is true could be determined only by eliminating the direct stimulation without affecting vision, and no convenient way of doing this has been found. Illumination of the eyes alone is almost impossible as long as the fish are free to move, while any restraint of the fish results in an excitement which itself has very marked effects on the condition of the melanophores.

It is, however, a relatively simple matter to remove the eyeballs and thus eliminate the response to background. Tests with 12 fish thus blinded showed that the melanophore change of which they were capable in response to differences in illumination was, at best, not more than half as great as that of normal fish. Such blinded fish appeared to be maximally dark at light intensities of 1 or 2 foot-candles or more. Reduction of the light produced a partial melanophore contraction, so that on a black background at intensities of 0.1 and 0.01 foot-candle the blinded fish were usually less dark than normal ones. Even in the complete absence of light, however, the appearance of the blinded fish could best be described as "moderately dark." Normal fish on a white background, even at 0.01 foot-candle, were much more pale than any blinded fish was ever observed to become, and also more pale than they themselves would be in complete darkness. Thus, although the observed responses of normal fish may have been the result of several stimuli, it at least seems clear that the response to background was predominant even at the lowest intensity used.

It may still be asked why the low rate of change which does occur at low light intensity does not eventually result in the same amount of change as the rate prevailing at higher intensity, even though the time to reach the final condition might be longer. The answer probably is that, while photic stimulation ordinarily dominates and determines the condition of the melanophores, it is not the only force capable of affecting them. In the complete absence of light, melanophores may still change as a result of these other forces. Below a certain point, the influence of light is no longer completely dominant, and the condition of the melanophores is then determined by the sum of all the forces of the external and internal environment which are capable of affecting them, and perhaps by the most stable condition inherent in the structure of the melanophores themselves. The weaker the light, then, the more closely will the equilibrium condition of the melanophores approach the one attained under similar conditions in the complete absence of light, and the more closely will the time required for the change to this condition approach the time required for the corresponding change in the absence of light.

SUMMARY

The melanophore responses of *N. biguttatus* to white or black background with illumination from above have been found to be approximately constant in rate and amount at light intensities of 100, 10, and 1 foot-candle.

A decrease in both the rate and amount of melanophore change appeared at an intensity of 0.1 foot-candle, and a further such decrease at 0.01 foot-candle. These changed responses are thought to be due to the incomplete stimulation of the visual elements by such low illumination.

Although the influence of the background still appeared to be most important, the response on either white or black background at these low intensities began to approach the response to complete darkness, namely, a slow change to a condition of partial expansion of the melanophores.

LITERATURE CITED

BROWN, F. A., JR. 1936. Light intensity and melanophore response in the minnow, *Ericymba buccata* Cope. Biol. Bull., 70:8–15.

DUSPIVA, FRANZ. 1931. Beiträge zur Physiologie der Melanophoren von Fischembryonen. Sitz.-ber., Akad. d. Wiss., Wien, mathem.-naturw. Kl., 140:553–96.

KOLLER, G. 1934. Über den Farbwechsel von Coregonenlarven. Biol. Centralbl., 54:419–36.

MAST, S. O. 1914. Changes in shade and pattern in fishes and their bearing on the problems of adaptation and behavior. Bull. U.S. Bur. Fisheries, 34:177–238.

MURISIER, P., 1921. Le Pigment mélanique de la truite. Rev. suisse de zool., 28:45–98, 149–96, 243–300.

POUCHET, G. 1876. Des changements de coloration sous l'influence des nerfs. Jour. anat. et physiol., 12:1–90, 113–65.

SUMNER, F. B. 1911. The adjustment of flatfishes to various backgrounds. Jour. Exper. Zoöl., 10:409–505.

THE SIMILARITY BETWEEN THE HYPOPHYSEAL CHROMATOPHOROTROPIC HORMONE AND THE CHROMATOPHOROTROPIC HORMONE OF THE CRUSTACEAN EYESTALK

(One plate)

A. A. ABRAMOWITZ
From the Marine Biological Laboratory, Woods Hole, Massachusetts
and the Biological Laboratories, Harvard University

THE discovery of Smith (1916) and Allen (1916) that extirpation of the hypophyseal anlage in embryos of the tadpole was followed by striking changes in the pigmentary coat of the developing animals led to a complete revision of the problem of chromatic activity. This, and especially the subsequent work of Atwell (1919), Swingle (1921), Krogh (1922), and Hogben (1924), demonstrated quite convincingly that the pituitary gland secretes a hormone which controls specifically the expansion of the integumentary melanophores of the amphibians, and thus delivered the first blow to Pouchet's conception (1876), which was generally held prior to 1915, that melanophore reactions of animals were governed through the intervention of peripheral nerves. Since this date it was found in rapid succession that the chromatophores of crustaceans (Perkins, 1928; Koller, 1928), most elasmobranchs (Lundstrom and Bard, 1932), cyclostomes (Young, 1935), and some reptiles (Noble and Bradley, 1933; Kleinholz, 1935) were controlled by hormones.

The teleosts and possibly also the African chameleons (although the latter have not yet been investigated) do not lose completely their ability to respond to black backgrounds following hypophysectomy (von Frisch, 1911; Desmond, 1924; Matthews, 1933· Parker, 1934; Abramowitz, 1936*a*, 1937*d*). The comparative physiology of these two groups whose melanophores appear to be controlled chiefly by the autonomic nervous system has been discussed in detail by Sand (1935), but there has been no similar comparative treatment of the melanophore reactions of those animals whose color changes are due directly to the intervention of blood-borne hormones. The experiments and results which have been published in abstract form elsewhere (Abramowitz, 1936*b*, *c*, *d*) form the foundation for a comparative treatment of the endocrine basis of pigmentary activity in the crustaceans and the lower vertebrates, and serve to point out certain similarities between the chromatophorotropic hormones of these two groups. Professor Parker's book (1936), which integrates in terms of neurohumoralism the older concepts of nervous and endocrine mechanisms relating to chromatophores, is especially valuable for a unified concept of the entire field of chromatic physiology.

MATERIALS

1. *The chromatophores.*—Lower vertebrates possess four kinds of chromatophores which are single cells and are always monochromatic. These are the melanophores, containing melanin; guanophores, containing pigments derived from the purines; xanthophores and erythrophores, whose pigments belong to the carotenoid series. Crustacean

chromatophores, however, are not usually single cells but are composed of two or more cells, somewhat like a coenocyte (Keeble and Gamble, 1904). Furthermore, crustacean chromatophores may be monochromatic, like those of the vertebrates, or polychromatic, in which case two or more pigments are present within the same chromatophore. Chemically, pigments similar to those mentioned occur in the crustaceans. However, the pigments of the Crustacea cannot be positively identified by their colors, for the melanins range from yellow through red to black, while the polyene pigments also range from yellow to dark brown in color. Consequently, certain crustacean chromatophores which have been designated as melanophores may in reality be "carotenophores," and vice versa.

It appears that the chromatophores of the vertebrates may be compared only with the monochromatic chromatophores of the Crustacea, if with any at all, and that only chromatophores containing similar pigments may be safely compared with one another. Thus, the melanophores of vertebrates may be compared with monochromatic melanophores of crustaceans, the xanthophores with the monochromatic chromatophores containing a similar pigment in the crustaceans, etc. The polychromatic chromatophores of the crustaceans, it appears, must be treated as a special type and cannot be compared fairly with any chromatophore of the vertebrates, for nothing anatomically similar to these polychromatic chromatophores occurs in the vertebrates. Whenever melanophores occur abundantly in either the crustaceans or the vertebrates, they are usually the chief instruments in provoking color changes. In this paper the melanophores were studied almost exclusively in the brachyuran crustaceans and in such vertebrates as the frog, the lizard, the dogfish, and several teleosts.

2. *The hormones.*—The vertebrate chromatophorotropic principle is found in the pituitary gland from cyclostomes through primates. It has been termed the "melanophore hormone" (Smith and Graeser, 1924; Jores, 1932; Dietel, 1932; Stehle, 1936; to mention a few), the "'B' substance" (Hogben and Slome, 1936), and "intermedin" (Zondek and Krohn, 1932*a*). This principle affects not only vertebrate melanophores (Hogben, 1924) but also erythrophores (Abolin, 1925; Saphir, 1934; Zondek and Krohn, 1932*a*, *b*) and xanthophores (Abolin, 1925; Giersberg, 1932; Abramowitz, 1936*e*). The possibility that there are separate hormones for each of these three chromatophores exists (cf. editor's footnote, Zondek, 1935*a*, *b*), and, in fact, the separate identities of a melanophore and an erythrophore hormone have been advocated (Jores and Lenssen, 1933; Rodewald, 1935). This has, however, been denied by Böttger (1934, 1936). Consequently, it will be perhaps best, for the present, to regard the chromatophorotropic principle as a hormone which affects all three chromatophores, until it is shown conclusively that more than one hormone is involved. The most suitable of the names for this hormone, therefore, is "intermedin," since this term does not necessarily refer to an effect on any particular type of chromatophore.

The relation of intermedin to other hypophyseal principles seems to be clearly indicated. Zondek and Krohn (1932*d*) claimed their intermedin had no anterior or posterior lobe hormones, but antidiuretic activity has been found (Sulzberger, 1933; Turner, 1935). Downes and Richards (1935) achieved a partial separation of antidiuretin and intermedin. Finally, Fisher (1936) reported that atrophic pars nervosa lacks vasopressin, antidiuretin, and oxytocin, but not the chromatophore factor. It appears, therefore, that intermedin is a distinct hormone of the hypophysis. However, the vasopressor and antidiuretic effects may be due to one hormone (Fraser, 1937).

The hormone of the crustacean eyestalk has been called the "eyestalk hormone" (Perkins, 1928), the "crustacean chromatophore activator" (Kropp and Perkins, 1933), and the "crustacean chromatophorotropic hormone" (Abramowitz, 1937*b*). All these general terms are applicable because the eyestalk extract affects the erythrophores, melanophores, guanophores, and "xanthophores" of the Crustacea. Neither vertebrate nor invertebrate pigmentary hormone has been isolated in chemically pure form.

The terms "intermedin" and "eyestalk hormone" will be used throughout this paper. All experiments with intermedin were performed with dilutions of one stock solution prepared from commercial sheep (whole gland) pituitary powder. Oxytocic and vasopressor activity are negative, but some antidiuretic effect was found by testing normal frogs. Quantitative treatment is obtained by expressing the amount of intermedin injected in terms of gram equivalents of the powder. Eyestalk hormone was prepared as a water extract of the eyestalks, boiled to coagulate the proteins, or, in some cases, in more purified form (Abramowitz, 1936*b*).

EXPERIMENTS AND METHODS

I. THE COMPARATIVE PHYSIOLOGY OF MELANOPHORE RESPONSES; THE RELATIONS AMONG THE EYES, ENDOCRINE GLANDS, AND THE MELANOPHORES

All investigators are agreed that the eyes are essential in initiating melanophore responses of animals, such as are induced by placing the animal over illuminated white or black backgrounds. Yet the relation between the eyes and the pituitary in the vertebrates and the eyes and the eyestalk glands of the Crustacea has not been definitely solved. This relationship can be analyzed by performing similar surgical operations. When the eyes are removed from such representative specimens as the frog, the lizard, the dogfish, and the lamprey, the melanophores of the integument become expanded and remain in a more or less expanded state indefinitely, provided illumination is present and the temperature is maintained normal for each particular specimen. When a similar operation is performed on the eyestalks of the brachyurans by excision of only the retina, the same result is obtained, although in this case the existence of a diurnal rhythm modifies the result (Abramowitz, 1937*b*). The expansion of the melanophores following excision of the retina has been thought by Parker, Brown, and Odiorne (1935) to be due to an innate property of these chromatophores. However, this does not seem to be valid, because, if such blinded vertebrates are now hypophysectomized, complete and enduring pigment concentration ensues. Similarly, if the remainder of the eyestalks in which the endocrine glands are located is excised, the melanophores of the brachyurans become and remain contracted. Thus, it seems that, when the eyes are removed in the vertebrates, the pituitary secretes intermedin continuously under the specified environmental conditions because a mechanism (eyes) which can completely inhibit the release of the hormone is removed.[1] A similar situation is found in the brachyurans, where excision of the retina produces a continuous secretion of the chromatophorotropic hormone for 3 or 4 days before the diurnal rhythm is re-established. The entire situation is summarized in Tables 1 and 2.

This similarity in the behavior of melanophores was pointed out by the author (1935), and led to the studies now being reported. It seems clear that the reactions of the melanophores of the Brachyura and of the vertebrates as listed in Table 1 are identical following the removal of the chromatophorotropic hormone from the circulation. Administration of the hypophyseal chromatophore hormone easily restores the dark coloration in hypophysectomized frogs (Hogben, 1924), lizards (Kleinholz, 1935), dogfishes (Lundstrom and Bard, 1932), and lampreys (Young, 1935). Administration of the eyestalk hormone to blinded brachyurans also restores the dark coloration (Carlson, 1935; Abramowitz, 1936*c*). Thus, the responses of the melanophores of the brachyurans and of the vertebrates cited to the chromatophore hormone of each group, respectively, are also identical. The possibility that the identical reactions might be due to a similarity in the hormones involved was next investigated.

---

[1] In the cyclostomes, the pineal complex supplements the eyes in inhibiting the release of melanophore hormone (Young, 1935). In this connection it might be pointed out that a light receptor in the skin may aid in keeping blinded animals dark by stimulating the pituitary.

TABLE 1

CONDITION OF MELANOPHORES AFTER REMOVAL OF THE EYES AND GLANDS

Author	Species or Genus	Condition	State of Melanophores
Megašur, 1912	*Uca pugnax*	Eyestalks extirpated (glands and eyes removed)	C Invertebrates Brachyura
This paper	*U minax*		C
Abramowitz, 1935	*Portunus anceps*		C
Carlson, 1935	*U pugilator*		C
This paper	*Mustelus* (pups)	Hypophysectomized +eyeless	C Vertebrates Elasmobranch
This paper	*Rana*		C Amphibian
Kleinholz*	*Anolis*		C Reptile
Hogben and Slome, 1931	*Xenopus*		C Amphibian
Young, 1935	*Lampetra*		C Cyclostome

Symbols C=concentrated; *=personal communication

TABLE 2

SUMMARY OF THE EFFECTS OF GLAND OR EYE REMOVAL

Animal	Condition with Illumination and Normal Temperature	State of Melanophores
Vertebrates	Eyes and hypophysis removed	C
	Eyes intact, hypophysis removed	C
	Eyes removed, hypophysis intact	E
Invertebrates (Brachyura)	Retina and glands removed (eyestalk extirpation)	C
	Retina intact, glands removed	?
	Retina removed, glands intact	E

Symbols C=contracted, E=expanded, ?=has not been performed

II. EFFECTS OF THE EYESTALK HORMONE ON VERTEBRATE CHROMATOPHORES

Koller and Meyer (1930) and Meyer (1931) showed that eyestalk extracts of *Crangon* contracted the melanophores of *Gobius* and *Pleuronectes*, while rostral organ extracts expanded these cells. Perkins and Kropp (1932*a*, *b*) and Kropp and Perkins (1933) found that extracts of *Crago* produced melanophore contraction in *Anguilla*, *Myoxocephalus*, and *Limanda;* no response in *Pollachius* and *Scomber;* and melanophore expansion in *Clupea*. Extracts of the eyestalks of *Pagurus* and *Homarus* produced melanophore contraction in *Fundulus*. Furthermore, these authors found that stalk extracts of *Palaemonetes*, *Callinectes*, *Homarus*, or *Pagurus* produced melanophore expansion in white-adapted tadpoles.

1. *Amphibians.*—Kropp and Perkins' results on normal white-adapted frog tadpoles were first verified. Ten normal white-adapted tadpoles were injected intraperitoneally with an extract of *Palaemonetes* eyestalks. The injection volume was 0.1 cc., and the concentration of hormone was 50 E.S./cc.[2] Melanophore expansion occurred in 9 animals. Similar injections into normal black-adapted specimens brought about no change in the already expanded melanophores. About 30 hypophysectomized specimens[3] were injected, with results similar to those of the white-adapted group (Pl. I, Figs. 1 and 2). The normal tadpoles were maintained on the same background after making the injection as before, a procedure which is standard for all experiments in which normal animals are used.

The same experiment was repeated with adult frogs (*Rana pipiens*). One-half cubic centimeter of a purified extract of 100 E.S./cc. (*Palaemonetes*) was injected into the dorsal lymph sac. Five white-adapted, 5 black-adapted, and approximately 20 hypophysectomized frogs were tested, and the results described for the tadpoles were verified (Pl. I, Fig. 3). The injected pituitaryless specimens remained dark, in some cases, for 10 hours. Moreover, responses could be elicited with injections of as little as 10 or 15 E.S. (about 3 *Uca* units). Frog melanophores are exceedingly sensitive to intermedin, for darkening occurs in hypophysectomized specimens after injection of 0.01 mg. equivalent.

2. *Elasmobranchs.*—As test objects, the pups of the smooth dogfish, *Mustelus canis* were used. Extracts of the eyestalks of *Libinia* and *Callinectes* were tested. A dose of 20 E.S. was found to produce complete expansion of the melanophores of hypophysectomized pups (eight tests) when injected intramuscularly. Intraperitoneal injections were negative. The animals which responded positively were injected near the anterior dorsal fin, and the darkening that appeared usually enveloped most of the anterior half of the fish. The same dosage tested similarly on 4 normal black-adapted specimens produced no change in the expanded melanophores. The expansion of melanophores in the hypophysectomized series persisted for 5 hours. The dosage of the crustacean hormone is rather high (about 80 *Uca* units), but the minimum dosage of intermedin injected intraperitoneally necessary to darken hypophysectomized pups was found to be 0.1 mg. equivalent (20 animals tested).

3. *Reptiles.*—Unfortunately, only few experiments were performed, owing to difficulties encountered in securing the lizard, *Anolis*. Through the kindness of Dr. L. H. Kleinholz, 7 *Anolis* were available. Three were hypophysectomized by a direct oral route (one dying the next day); and 4 normal animals, 2 white-adapted, and 2 black-adapted, were used as controls. These specimens were injected intraperitoneally with

---

[2] The letters "E.S " are used as an abbreviation for eyestalk.

[3] Two-thirds of these hypophysectomized tadpoles were kindly given to me for these experiments by Dr. W M. Etkin, of the American Museum of Natural History, New York, to whom I am much indebted. The remaining tadpoles were hypophysectomized at the 4-mm. stage by Allen's method (1916).

0.1 cc. of an extract of *Palaemonetes* in a concentration of 100 E.S./cc. The normal black-adapted specimens showed no changes. The normal white-adapted animals showed irregular dark areas (melanophore expansion) along the ventral surfaces, but the dorsal surface remained green (melanophore contraction). One of the hypophysectomized animals responded in a similar fashion, while the other showed a more uniform darkening (Pl. I, Fig. 4).

4. *Teleosts.*—The catfish, *Ameiurus*, and the killifish, *Fundulus*, were used as test objects. The catfishes were hypophysectomized by the opercular approach (Abramowitz, 1937*a*), and bands of caudal melanophores were denervated by Wyman's (1924) technique. One set of 6 animals was placed in white vessels for 5 days, and another set of equal number placed in black dishes. Two sets of normal animals were likewise placed in white or black dishes. Innervated and denervated melanophores of both normal and hypophysectomized specimens become adjusted to these backgrounds (Parker, 1934). In the white vessels, both innervated and denervated cells become contracted; while in black surroundings, the melanophores (innervated and denervated) of normal animals are fully expanded but those of hypophysectomized animals are not completely expanded (Abramowitz, 1936*a*).

Each animal was injected intraperitoneally with an extract of 100 E.S. of *Palaemonetes*, but the results were not clear cut. Within an hour there was no change in the black-adapted normal series, usually a slight expansion in the innervated and denervated areas of the white-adapted normal and hypophysectomized groups, and inconclusive changes in the black-adapted hypophysectomized group. The experiment was repeated by injecting a larger dose (200 E.S.) into 15 white-adapted normal fishes. Now the expansion of innervated and denervated cells was more marked; and a condition of half-expansion, remaining for 2 hours, was obtained in 7 animals. The remaining 8 animals darkened very strongly, but died during the experiment, since the excessive dose was undoubtedly toxic. The experiment was again repeated, using a more purified extract (Abramowitz, 1936*b*) and injecting a smaller dose intramuscularly in order to obtain a more local response. While this type of an injection is undoubtedly inferior, because subcutaneous injections in teleosts result often in nonspecific effects, it does avoid the use of a dose which is toxic. Accordingly, 24 fishes were divided into four groups as described above, with the exception that bands of cells were not denervated. Injections of 50 E.S. (*Palaemonetes*) were made into the dorsal muscles at a region slightly anterior to the anterior dorsal fin. After 15–20 minutes, melanophore expansion resulted in the two white-adapted groups and in the black-adapted hypophysectomized group but no change occurred in the normal black-adapted group.

It is difficult to conclude definitely that the eyestalk extract expands the melanophores of the catfish, since the necessary proof (complete expansion in a denervated area) was not obtained. At best, the denervated cells showed a condition of half-expansion. The reaction of the innervated cells might be due actually to a toxic action on the nervous system, though this could not account for the response of the denervated area. Repetition of this experiment with hypophysectomized *Fundulus*, a section of whose tails was denervated by Wyman's method, gave negative results. In no case did the crustacean extract, when injected intraperitoneally, produce melanophore reactions in either innervated or denervated melanophores.

The expansion of erythrophores of teleosts, such as *Phoxinus*, is claimed by Zondek and Krohn (1932*a*, *b*) to be a specific indication of the presence of intermedin. Although this is questionable, it was of interest to test the action of the crustacean hormone upon the erythrophores. No *Phoxinus* being available, the dace, *Chrosomus erythrogaster*, was used (Saphir, 1934; A. Young, 1934). Twenty-four fish were injected either subcutaneously or intraperitoneally. The injection volume was 0.1 cc. of a stock solution of 100 E.S./cc. (*Palaemonetes*). Seventy per cent of the animals injected subcutaneously showed erythrophore expansion (some also showed melanophore expansion), but intraperitoneal injections were rarely followed by a positive response.

Taking all of the results into consideration, it appears that the action of the crustacean extract on vertebrate melanophores is to induce generally the expanded state of these chromatophores. This action is characteristic of intermedin, and it seems that the effect of these two extracts on the melanophores is similar. Furthermore, the erythrophores of vertebrates tend to respond similarly. Information concerning the action of the crustacean hormone on vertebrate xanthophores would be exceedingly interesting.

III. EFFECT OF INTERMEDIN ON CRUSTACEAN CHROMATOPHORES

The brachyurans, *Uca pugilator* and *U. pugnax*, were chosen as test objects (Abramowitz, 1936*c*). Sixty animals, whose eyestalks were extirpated 2 days previously, were injected with various known doses of intermedin. These blinded animals are pale, regardless of light intensity or of background. The most suitable dose was found to be 0.05 cc. of a concentration of 1 gm. equivalent of intermedin in 1 cc. of sea water. Typical responses ensued (cf. Abramowitz, 1937*c*). The animals became dark within 1½ hours, remained dark for 7 hours, and finally became pale again (Pl. I, Figs. 5 and 6). Microscopic examination disclosed that intermedin produced expansion of the melanophores and contraction of the guanophores and "xanthophores." The erythrophores were unaffected, these remaining contracted. The eyestalk hormone of *Uca* (or of any other crustacean tested [cf. Abramowitz, 1937*c*]), when injected into blinded specimens, produces the same reaction except that the erythrophores become expanded. Thus, there is a significant difference between the qualitative effects of vertebrate and invertebrate pigmentary extracts in the crab, *Uca*.

IV. EFFECT OF EXTRACTS OF THE ASCIDIAN SUBNEURAL GLAND COMPLEX ON VERTEBRATE AND CRUSTACEAN MELANOPHORES

Because of its embryological history and cytological appearance, the subneural gland has been considered a primitive pituitary gland. Butcher (1930) demonstrated that the subneural gland contains the oxytocic hormone, and Bacq and Florkin (1935) and Abramowitz (1937*c*) showed that the chromatophorotropic principle is also present. The former produced melanophore expansion in normal frogs with an extract of *Ciona*, while the latter produced melanophore expansion in *Uca* with an extract of *Molgula* (subneural gland = 0.2 *Uca* units). The vasopressor hormone is also present in this primitive pituitary of protochordates (Bacq and Florkin); and recently Hogg (1937) has reported a gonadotropic effect of the subneural gland of *Polycarpa*, the large Bermudan form, on the immature mouse.

V. COMPARATIVE PROPERTIES OF THE CRUSTACEAN HORMONE AND INTERMEDIN

The properties of the two hormones may best be compared in tabular form (Table 3).

Intermedin is potentiated from ten to twenty-five times by exposure to alkali (Hogben and Winton, 1922; Smith and Graeser, 1924; Zondek and Krohn, 1932*d;* Böttger, 1934;

Jores, 1934; Stehle, 1936). The eyestalk hormone is stable to NaOH when a mixture of the hormone and NaOH is boiled for 5 minutes (Carlson, 1936) but not for longer periods (Abramowitz, 1937*b*). If the potentiation by alkali is due to a change in the intermedin molecule (Stehle, 1936), it would seem that there is a significant chemical difference between the invertebrate and vertebrate hormones. However, the potentiation may be due to the removal of an inhibiting substance (Böttger, 1936) or to circulatory facilitation resulting from the destruction of vasopressin by alkali (Hogben and Gordon, 1930).

DISCUSSION

The general trend of the evidence seems, therefore, to indicate a similarity between the vertebrate and invertebrate pigmentary hormones. This similarity between the eyestalk hormone and "pituitary secretion" was first pointed out by Kropp (1932), inasmuch as both eyestalk extracts and pituitrin expanded tadpole melanophores. Additional evidence has been presented in this paper from the comparative physiology of melanophore responses, from some chemical and physical properties of the two hormones, and from reciprocal replacement therapy tests. Nevertheless, while the similarity seems to be striking in several respects, one must approach the problem with a good deal of caution.

TABLE 3

	Crustacean Eyestalk Hormone (Abramowitz, 1937*b*)	Intermedin (Zondek and Krohn, 1932*d*)
Water	Soluble	Soluble
Ethanol (absolute)	Soluble	Soluble
Ethyl ether	Insoluble	Insoluble
Chloroform	Insoluble	Insoluble (practically)
Acetone (absolute)	Insoluble	Insoluble
Benzine	Insoluble	Insoluble
Methanol (absolute)	Soluble	Soluble
Acid (HCl)	Stable	Stable
Alkali (NaOH)	Stable	Stable
Boiling	Stable	Stable
Oxidation	Destroyed	Destroyed

In the first place, the evidence from the comparative physiology of melanophores may be a peculiar coincidence whose significance is quite different from that herein described. Two significant exceptions must be noted. The black pigment (a melanin) of *Crangon* expands when the eyestalk glands are removed (Koller, 1930), and contracts when the animal is injected with the eyestalk hormone. However, it becomes dispersed when *Crangon* is injected with hypophysin (Böttger, 1934). Thus, the two extracts produce opposed reactions. The full significance of these observations cannot be fully appreciated at present because the black pigment of *Crangon* is contained mainly in polychromatic chromatophores. The other case is that of *Ligia* (Kleinholz, 1937). *Ligia* has monochromatic melanophores (single cells!) which contract when its hormone is injected. This is, of course, opposite to the behavior of the melanophores of the brachyurans listed in Table 1. The action of intermedin or of the brachyuran eyestalk on *Ligia*'s melanophores would be well worth investigating in this connection.

The reliability of the reciprocal replacement therapy experiments is undermined by the existence of nonspecific responses of the melanophores. This danger, one of the greatest in chromatophore research, has not been duly emphasized, for chromatophores are unusually sensitive structures and may be induced to respond to an array of chemical and physical stimuli. It is generally acknowledged that intermedin is a specific hormone for vertebrate chromatophores. Extracts of tissues other than the pituitary gland do not produce melanophore expansion in a hypophysectomized specimen (Hogben, 1924). The same may also be true of the brachyuran and other crustaceans with respect to the eyestalk glands. Yet the possibility exists that the eyestalk hormone produces only a nonspecific response when injected into a hypophysectomized vertebrate, and vice versa. This question cannot be definitely answered at present, for there is still little known concerning the existence of nonspecific melanophore responses in hypophysectomized vertebrates or eyestalk-amputated brachyurans.[4]

The qualitative differences, already mentioned, in the reciprocal actions of the pigmentary hormones cannot be properly explained. The failure of intermedin to expand the erythrophores of the crab, *Uca*, should be investigated further, and the reactions of the melanophores in *Crangon* are still uncertain. Consequently, these differences cannot be discussed adequately until more observations are at hand. From the quantitative standpoint, only the response of the hypophysectomized frog to eyestalk extract is similar to that resulting from a weak dosage of intermedin. The action of intermedin in *Uca* in the concentration used is indistinguishable quantitatively from that of an eyestalk extract containing 2 *Uca* units. However, this dose of intermedin is significantly high. Greater dilutions usually produced no reactions; furthermore, I have been unable to balance comparable doses by testing frogs and crabs simultaneously. In other words, 0.05 gm. equivalents of intermedin are quantitatively equal in effect on the crab to 2 *Uca* units, but 2 *Uca* units of *Palaemonetes* hormone are ineffective usually on the hypophysectomized frog, while 0.05 gm. equivalents of intermedin will darken frogs for several days. Much more pituitary hormone is needed, then, to produce a reaction in crabs than in frogs, and vice versa. Assuming that the reciprocal effects are due to the hormones and are not results of toxicity or contamination of the extracts, the explanation of this difference may be: (1) a difference in the responsive tissues, since a crustacean melanophore and a vertebrate melanophore may be as different physiologically as they are anatomically, (2) a difference in the chemical composition of the hormones or impurities present in the extract.[5]

Concerning the first, it can be pointed out that the melanophores of *Uca* do not contract to adrenalin, while those of fish and amphibians, as is well known, react to very small doses. Furthermore, the melanophores of some selachians are refractive to adrenalin, while in other elasmobranchs adrenalin may produce pallor indirectly, through vasoconstriction (Wykes, 1936). It is obvious, then, that vertebrate melanophores do not always react by contracting to adrenalin hydrochloride; and, indeed, the reaction, at least in one particular case (*Anolis* [Kleinholz, 1936]), may be just the reverse. Vertebrate melanophores, therefore, may be slightly different physiologically from one genus to another.

---

[4] Another point to be considered is the problem of the number of pituitary chromatophorotropic hormones. The recent work of Hogben (1936) and of Hogben and Slome (1936) points to two pituitary pigmentary hormones—an expanding (intermedin, "B" substance) and a contracting ("W" substance) hormone. The previous discussion has proceeded on the assumption that only one hormone (intermedin) is involved in the chromatic physiology of amphibians and other vertebrates, simply because the bulk of this work had been completed before the most recent papers of Hogben and of Hogben and Slome had appeared. Moreover, the interpretations of most of the data, already presented, would not be seriously invalidated by the existence of the "W" hormone, since only the expanding hormone is under discussion.

[5] A third possibility is that both the hormones and the melanophores of brachyurans and vertebrates are different but that the mechanism by which intermedin expands a vertebrate melanophore may be similar to that by which the eyestalk hormone expands a crustacean melanophore. There is, however, nothing known concerning the microphysiology of the melanophores under endocrine influences.

The second possibility cannot be adequately discussed because neither of the two hormones has been obtained in a pure state. The word "similarity" has been used throughout this paper to mean a similarity in physiological effect, not a chemical similarity or identity. The activity of both is found in more or less the same solvents, and some other properties are common to both; consequently, on this basis they may be said to behave alike. Judging from the chemistry and physiological effects of the sex hormones, for example, it would be extremely surprising to find chemically identical pigmentary hormones in the crustacean eyestalk, the ascidian subneural gland, and the vertebrate pituitary. Intermedin may even be slightly different chemically in the pituitary of different vertebrates. What may be possible, however, is that the vertebrate and invertebrate hormones may be related sufficiently to produce reciprocal pigmentary reactions when given in proper doses.[6]

SUMMARY

The monochromatic melanophores of the brachyurans remain expanded after loss of the eyes but contract after removal of the eyestalks. These responses are similar to those of the melanophores of amphibians, reptiles, elasmobranchs, and cyclostomes following optic enucleation and hypophysectomy, respectively. By reciprocal replacement therapy eyestalk extracts of several crustaceans have been shown to be effective in expanding melanophores of an elasmobranch (*Mustelus*), a teleost (*Ameiurus*), an amphibian (*Rana*), and a reptile (*Anolis*). Similarly, intermedin, the hypophyseal hormone, was found to produce an expansion of the melanophores of eyestalk-amputated brachyurans (*Uca*). The comparative chemical properties of the two pigmentary hormones are listed and discussed.

LITERATURE CITED

ABOLIN, L. 1925. Arch. f. mikr. Anat., 104:667.
ABRAMOWITZ, A. A. 1935. Proc. Nat. Acad. Sci., 21:677.
———. 1936*a*. Biol. Bull., 71:259.
———. 1936*b*. Proc. Soc. Exper. Biol. and Med., 34:714.
———. 1936*c*. Proc. Nat. Acad. Sci., 22:521.
———. 1936*d*. Anat. Rec., 67:108.

---

[6] Whether or not crustaceans other than brachyurans will respond to intermedin is not fully known. Dr. Kleinholz and I have found that intermedin contracts the red and yellow pigments of *Palaemonetes* in approximately 50 per cent of the cases. Furthermore, intermedin did not affect the retinal pigments of *Palaemonetes*. Eyestalk extracts, however, affect both body and retinal pigments of *Palaemonetes*. Consequently, *Uca* may be peculiar among crustaceans in that it responds definitely to intermedin preparations. This is an additional point which indicates that the similarity between the pigmentary hormones may not only be limited to several properties but may also be limited in scope to certain animal groups.

Abramowitz, A. A. 1936*e*. Amer. Nat., 70:372.

———. 1937*a*. Science, 85:609.

———. 1937*b*. Biol. Bull., 72:344.

———. 1937*c*. Jour. Exper. Zoöl., 76:407.

———. 1937*d*. Biol. Bull., 73:134.

Allen, B. M. 1916. Science, N.S., 44:1143.

Atwell, W. J. 1919. Science, 49:48.

Bacq, Z. M., and Florkin, M. 1935. Arch. internat. de physiol., 40:422.

Böttger, G. 1934. Zeitschr. f. vergl. Physiol., 21:414.

———. 1936. Klin. Wchnschr., 15:73.

Butcher, E. O. 1930. Jour. Exper. Zoöl., 57:1.

Carlson, S. Ph. 1935. Proc. Nat. Acad. Sci., 21:549.

———. 1936. Kungl. Fysiogr. Säll., 6:1.

Desmond, W. F. 1924. Anat. Rec., 29:103.

Dietel, F. G. 1932. Klin. Wchnschr., 1:2075.

Downes, H. R., and Richards, L. 1935. Jour. Biol. Chem., 110:81.

Fisher, C. 1936. Endocrinology, 21:19.

Fraser, A. M. 1937. Jour. Pharmacol. and Exper. Therap., 60:82.

Frisch, K. von. 1911. Arch. ges. Physiol., 138:319.

Giersberg, H. 1932. Zeitschr. f. vergl. Physiol., 18:369.

Hogben, L. T. 1924. The pigmentary effector system. Edinburgh: Oliver & Boyd.

———. 1936. Proc. Roy. Soc. London, B, 120:142.

Hogben, L. T., and Gordon, C. 1930. Jour. Exper. Biol., 7:286.

Hogben, L. T., and Slome, D. 1931. Proc. Roy. Soc. London, B, 108:10.

———. 1936. *Ibid.*, 120:158.

Hogben, L. T., and Winton, F. R. 1922. Biochem. Jour., 16:619.

Hogg, B. M. 1937. Proc. Soc. Exper. Biol. and Med., 35:616.

Jores, A. 1932. Klin. Wchnschr., 2:2116.

———. 1934. *Ibid.*, p. 1269.

Jores, A., and Lenssen, E. W. 1933. Endokrinologie, 12:90.

Keeble, F., and Gamble, F. W. 1904. Phil. Trans. Roy. Soc. London, B, 196:295.

Kleinholz, L. H. 1935. Biol. Bull., 69:379.

———. 1936. Proc. Nat. Acad. Sci., 22:454.

———. 1937. Biol. Bull., 72:24.

Koller, G. 1928. Zeitschr. f. vergl. Physiol., 8:601.

———. 1930. *Ibid.*, 12:632.

Koller, G., and Meyer, E. 1930. Biol. Zentralbl., 50:759.

Krogh, A. 1922. The anatomy and physiology of the capillaries. New Haven: Yale University Press.

Kropp, B. 1932. Proc. Nat. Acad. Sci., 18:690.

Kropp, B., and Perkins, E. B. 1933. Biol. Bull., 64:226.

Lundstrom, H. M., and Bard, P. 1932. Biol. Bull., 62:1.

Matthews, S. A. 1933. Biol. Bull., 64:315.

Megašur, F. 1912. Arch. f. Entw 'mech., 33:462.

Meyer, E. 1931. Zool. Jahrb. Abt. allg. Zool. Physiol., 49:231.

Noble, G. K., and Bradley, H. T. 1933. Biol. Bull., 64:289.

Parker, G. H. 1934. Jour. Exper. Zoöl., 69:199.

———. 1936. Color changes in animals in relation to nervous activity. Philadelphia: University of Pennsylvania Press.

Parker, G. H.; Brown, F. A.; and Odiorne, J. M. 1935. Proc. Amer. Acad. Arts and Sci., 69:439.

Perkins, E. B. 1928. Jour. Exper. Zoöl., 50:71.

Perkins, E. B., and Kropp, B. 1932*a*. Mt. Desert Island Biol. Lab. Rept., No. 4.

———. 1932*b*. Biol. Bull., 63:108.

Pouchet, G. 1876. Jour. anat. et physiol., 12:1, 113.

Rodewald, W. 1935. Zeitschr. f. vergl. Physiol., 21:767.

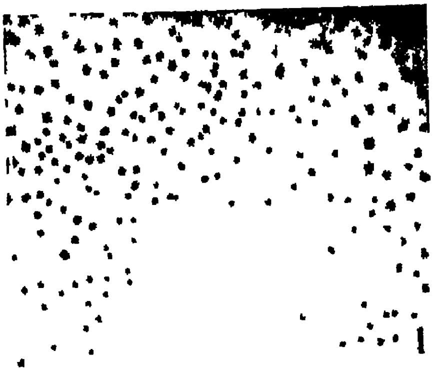
Sand, A. 1935. Biol. Rev., 10:361.

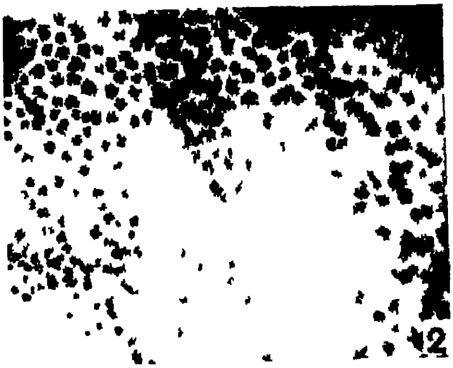
Saphir, W. 1934. Proc. Soc. Exper. Biol. and Med., 31:864.

Smith, P. E. 1916. Science, N.S., 44:280.

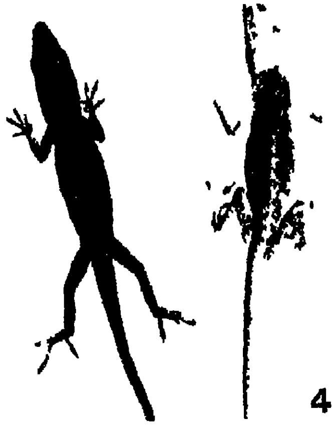
SMITH, P. E., and GRAESER, J. B. 1924. Anat. Rec., 27:187.
STEHLE, R. L. 1936. Jour. Pharmacol. and Exper. Therap., 57:1.
SULZBERGER, M. B. 1933. Jour. Amer. Med. Assoc., 100:1928.
SWINGLE, W. W. 1921. Jour. Exper. Zool., 34:119.
TRENDELENBURG, P. 1929. Die Hormone, Book I. Berlin: J. Springer.
TURNER, H. H. 1935. Endocrinology, 19:275.
WYKES, U. 1936. Jour. Exper. Biol., 13:460.
WYMAN, L. C. 1924. Jour. Exper. Zoöl., 39:73.
YOUNG, A. M. 1934. Amer. Jour. Clin. Path., 4:485.
YOUNG, J. Z. 1935. Jour. Exper. Biol., 12:254.
ZONDEK, B. 1935*a*. Glandular physiology and therapy, chap x. Chicago: American Medical Association.
———. 1935*b*. Jour. Amer. Med. Assoc., 104:637.
ZONDEK, B., and KROHN, H. 1932*a*. Naturwissenschaften, 20:134.
———. 1932*b*. Klin. Wchnschr., 10:405.
———. 1932*c*. *Ibid.*, 11:849.
———. 1932*d*. *Ibid.*, p 1293.

PLATE I


1


2

3


4

5

6

PLATE I

FIG. 1.—Melanophores of a hypophysectomized tadpole ×38.

FIG. 2.—Melanophores of a hypophysectomized tadpole after injection of eyestalk hormone. ×38.

FIG. 3.—*Left*, hypophysectomized frog injected with crustacean hormone; *center*, hypophysectomized frog injected with intermedin; *right*, hypophysectomized frog uninjected. × ⅓

FIG. 4.—*Left*, hypophysectomized *Anolis* injected with crustacean hormone; *right*, hypophysectomized *Anolis* uninjected. ×½.

FIG. 5.—*Top*, dark coloration produced in blinded crab (*U. pugilator*) injected with intermedin; *bottom*, pale blinded crab, uninjected ×1.

FIG. 6.—*Top*, *U. pugnax*, blinded, uninjected, *bottom*, *U. pugnax*, normal dark phase. ×½.

ON THE MOTOR AND INHIBITORY INNERVATION OF THE LEGS OF BRACHYURAN DECAPODS

(Two figures)

C. A. G. WIERSMA AND GEORGE MARMONT
William G. Kerckhoff Marine Laboratory of the California Institute
of Technology, Corona del Mar, California

VAN HARREVELD and Wiersma (1937) have described the innervation of the muscles of the cheliped of the crayfish *Cambarus clarkii* and *Astacus trowbridgii*, giving a description of the number, the course, and the function of the exciting and inhibiting axons going to the distal six muscles. They had previously found, for the same animals (1936), that the adductor of the claw is innervated by two motor fibers; stimulation of the thicker of these fibers produces a "fast" contraction, while stimulation of the thinner gives rise to a "slow" contraction. It was found that this double motor innervations occurs generally in other muscles of the legs of these animals as well as in those of all marine decapods investigated on this point (Wiersma and van Harreveld, 1938). Indeed, it was concluded, in the paper first mentioned above, that the muscles of the crayfish limb are commonly innervated by two motor axons and an inhibiting one (triple innervation).

In continuation of the foregoing studies the innervation of the legs of several brachyurans has been physiologically investigated. It will be shown that, although the triple innervation is also the rule here, there are, as compared to the innervation of the crayfish, some important differences both in the course and function of the inhibiting fibers (see the scheme shown in Figure 2). Those differences should be taken into account in investigations of the peripheral nerve muscle system of crabs.

METHOD

After removing a cheliped or a walking leg from the animal, the meropodite was opened and the nerve running through it was exposed by removal of the flexor and extensor muscles contained in the meropodite. Using the same technique as described in previous papers, the exciting and inhibiting axons were separated and tested by means of two pairs of micromanipulated electrodes, the nerve being submerged either

Subtribe	Superfamily	Family	Species Used
Oxystomata		Leucosiidae	*Randallia ornata* (Randall)
Dromiacea			No species obtainable
Brachygnatha	Oxyrhyncha	Parthenopidae Inachidae	*Heterocrypta occidentalis* (Dana) *Loxorhynchus grandis* Stimpson
	Brachyrhyncha	Cancridae Grapsidae	*Cancer anthonyi* Rathbun *Pachygrapsus crassipes* Randall

in sea water or in Pantin's marine solution when not being stimulated. In some cases the contractions were registered, but usually they were only visually observed. Whenever possible, measurements of the diameters of the fibers were made.

Five species of crabs were studied. Their place in the tribe Brachyura is indicated in the classification on the preceding page (after Borradaile).

EXPERIMENTS ON THE ADDUCTOR AND FLEXOR

When the whole leg nerve, prepared as indicated, was placed on the electrodes and faradically stimulated, often no contraction of any muscle was observed upon a stimulation strength which should have been adequate. Very strong stimulation, however, would cause closing of the claw and flexing of the propodite. Sometimes a very narrow range of weak stimuli would produce a contraction, while somewhat stronger stimuli would give no effect. After the nerve had been divided into several bundles, closing of the claw could usually be obtained from one of these. Further division of this bundle always yielded two fibers which were responsible for this contraction. In agreement with the results found by Wiersma and van Harreveld (1938), stimulation of the thinner fiber at frequencies below 50 per second usually produced a contraction which was more rapid than that resulting upon stimulation of the thicker fiber under the same conditions. As the frequency was raised above 50 per second, a transition frequency was found at which the two types of contraction were quite similar in appearance; at still higher frequencies, the adduction elicited by means of the thicker axon was always faster than that caused by the thinner one. The transition frequency was rather inconstant in the present experiments, varying not only with the species but also with the state of fatigue of the preparation. Since it is likely that the quick fatigue is a feature of the summer season, a more detailed comparative study of the transition frequency was not made at this time.

Upon simultaneous stimulation with one pair of electrodes of all the fiber bundles remaining after isolations of the two adductor motor axons, one or both of the latter axons being also stimulated with the other pair of electrodes, it was found that usually the contraction of the adductor was either suppressed entirely or reduced considerably. Next, the bundle responsible for this inhibition was isolated and divided. When the inhibiting axon was found, excitation of the remaining fibers of this bundle produced flexion of the propodite, an effect which had not been visible before. Further isolation yielded two fibers of different diameters which caused this contraction, indicating the existence of a double motor innervation here. These two motor fibers usually run quite close to the inhibiting axon mentioned above. When this inhibiting fiber was stimulated simultaneously with one or both of the flexor motor fibers, it was found that flexion inhibition also occurred; in other words, the adductor and the flexor have one inhibitory fiber in common. This fiber, then, is able to suppress or reduce the contractions produced by excitation of any one of four motor fibers.

In the large walking legs of *Loxorhynchus* it was possible to record the contractions of the adductor and of the flexor separately, the tendon of the latter muscle being connected directly to a lever, while the contraction of the adductor was recorded with a lever connected to the dactylopodite. Figure 1 shows the effect of stimulation of the inhibitor axon upon the contractions produced by the stimulation of the four motor fibers simultaneously. However, such records were difficult to obtain because the necessary stimulation of the inhibitor fiber during its preparation often resulted in fatigue of inhibition before isolation of the inhibitor had been accomplished.

EXPERIMENTS ON THE ABDUCTOR AND EXTENSOR

In the crayfish the flexor of the propodite has its own inhibitory axon, while the slow contraction of the adductor is inhibited, though to a slight extent, by the same fiber which inhibits the contraction of the extensor of the propodite (Hoffman, 1914; Serkoff, 1936; Marmont and Wiersma, unpublished). In crabs, however, the function of the extensor inhibitor was found to be restricted to the extensor muscle. As in the crayfish, the abductor and the extensor are innervated by a single common motor axon, but preparation of this motor axon and of the extensor inhibitor fiber is more difficult in crabs because the inhibitor fiber of the abductor runs in the same bundle. This means that no contraction of either muscle can be obtained until at least one of these three fibers has been separated from the others. This difficulty is mitigated by the facts that these rather large axons run in a bundle containing only a few small sensory fibers and that the bundle occupies a fairly constant position in the whole nerve. Since the extensor and the abductor muscles have one single motor fiber in common, they possess only one type of contraction and must be considered, as in the crayfish, as parts of one motor unit, being separately inhibitable.

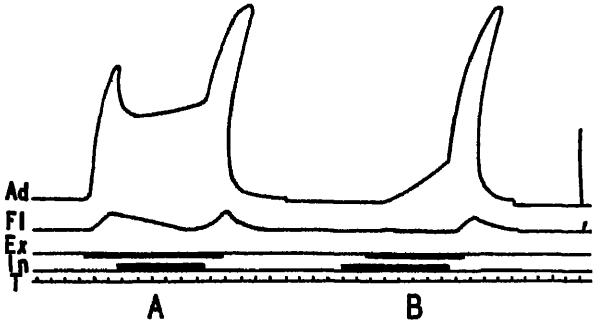


FIG. 1.—Preparation from *Loxorhynchus grandis* showing simultaneous inhibition of the contractions of the adductor and of the flexor of the propodite produced by stimulating their common inhibiting axon. The two motor fibers to the adductor and the two to the flexor are faradically stimulated on one pair of electrodes; the inhibitor is similarly stimulated by another pair. In *A*, inhibition is applied in the midst of a contraction; in *B*, the inhibition is started before excitation. In this preparation adductor inhibition is not complete. *Ad* is the isotonic contraction of the adductor; *Fl*, that of the flexor; *Ex* and *In* show the periods when excitation and inhibition were given, respectively; *T* is the time in seconds.

SCHEME OF INNERVATION

In Figure 2 the innervation to the four distal muscles found by this method in all the species of crabs investigated is compared to that found in the crayfish. It will be seen that the number of fibers is the same in both types, not only in total number but also as divided according to the function: "fast," "slow," or inhibitory. However, the following differences are present. In the crabs the opener inhibitor does not run in the bundle containing the adductor motor axons, at least not distally to the beginning of the meropodite. In these animals there is no extension of the extensor inhibitor to the closer muscle, as in the crayfish (we term such an extension the "X-fiber"), but instead there is an extension of the flexor inhibitor to the closer (Y-fiber). This Y-fiber is much more effective in producing inhibition of the adductor muscle than the X-fiber. It is interesting that in these two suborders of the decapods, whose limbs are morphologically quite similar, inhibition of the adductor should have been taken over in one group by the inhibitor of the flexor of the propodite and in the other group by the inhibitor of the extensor of the propodite

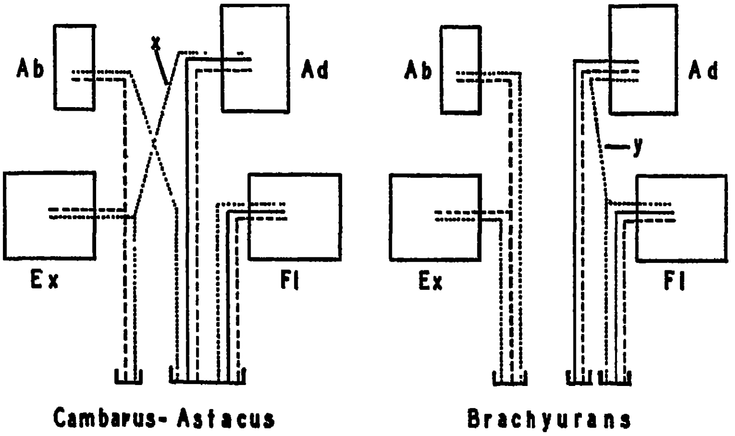


FIG. 2.—Diagrams of the innervation of the distal four muscles of the chelipeds or walking legs of the crayfish and of brachyurans. The unbroken lines represent an axon causing a "fast" contraction; the dashes represent those causing a "slow" contraction; and the dotted lines represent inhibiting axons. *Ab* is the abductor; *Ad*, the adductor; *Ex*, the extensor of the propodite; and *Fl*, the flexor of the propodite. *X* and *Y* mark the "X" and "Y" fibers.

Several anomuran decapods were given a preliminary investigation, namely, *Pagurus ochotensis*, Brandt, *Paguristes bakeri*, Holmes and *Blepharipoda occidentalis*, Randall. Physiologically, their innervation seemed identical with that occurring in the crayfish, except that no evidence was found for the existence of the X-fiber.

FIBER DIAMETERS

Measurements of the diameters of fibers from preparations of *Loxorhynchus*, *Cancer*, and *Pachygrapsus* are given in Table 1. As in the crayfish, the motor and the inhibitor axons are of large size, compared to the sensory axons. The diameter of any specific fiber bears no relation to the size of the species: the largest fibers occur in the walking legs of *Pachygrapsus*, which is one of the smaller species investigated. *Cancer* and *Loxorhynchus*, the largest species, had fibers of a diameter corresponding to those of the much smaller crayfish.

SUMMARY

The innervation of the legs of five species of brachyuran decapod crustaceans was investigated by isolating the motor and inhibiting axons going to the distal four muscles. The function of those fibers was observed upon stimulation.

As in the crayfish, the abductor and the extensor of the propodite muscles were found to be parts of one motor unit, but each possesses an inhibitor fiber; these three fibers run in the same bundle in the meropodite. The adductor and the flexor of the propodite each has its own "fast" and "slow" axons; however, only one inhibitory fiber is present, and this inhibitor is able to suppress or decrease contractions of both muscles.

TABLE 1

FIBER DIAMETERS

SPECIES	ADDUCTOR		COMMON INHIBITOR	FLEXOR		ABDUCTOR EXTENSOR	ABDUCTOR INHIBITOR	EXTENSOR INHIBITOR
	Fast	Slow		Fast	Slow			
Loxorhynchus:								
Claw........	30 μ	24 μ	21 μ	36 μ	27 μ	....	....	....
	42	30	....	....	....	....	....	....
	54	42	....	....	....	....	....	....
Leg.........	48	46	....	....	....	....	....	....
Cancer:								
Leg.........	45	36	....	....	....	42 μ	33 μ	36 μ
Pachygrapsus:								
Claw........	57	48	27	54	48	60	42	45
	60	42	....	....	....	54	45	42
Leg.........	84	51	....	....	....	60	45	45
	84	54	....	....	....	....	....	....
	66	42	36	66	40	....	....	....
	.....	....	....	60	....	45	42	42

LITERATURE CITED

HARREVELD, A. VAN, and WIERSMA, C. A. G. 1936. The double motor innervation of the adductor muscle in the claw of the crayfish. Jour. Physiol., 88:78.

———. 1937. The triple innervation of crayfish muscle and its function in contraction and inhibition. Jour. Exper. Biol. 14:448.

HOFFMAN, P., 1914. Über die doppelte Innervation der Krebsmuskeln. Zugleich ein Beitrag zur Kenntnis nervöser Hemmungen. Zeitschr. f. Biol., 63:411.

SERKOFF, PH. N. 1936. Elektrophysiologische Untersuchung der peripheren Hemmung im Scherenschliesser des Fluszkrebses. Pflügers Arch., 237:631.

WIERSMA, C. A. G., and HARREVELD, A. VAN. 1938. A comparative study of the double motor innervation in marine crustaceans. Jour. Exper. Biol. 15:18.

THE OLFACTORY REACTIONS OF DROSOPHILA MELANOGASTER MEIGEN TO THE PRODUCTS OF FERMENTING BANANA[1]

(Three Figures)

MELIA R. REED
Department of Zoölogy, University of Minnesota

THE most extensive early work on the olfactory sense of *Drosophila melanogaster* was that of Barrows (1907). In the course of his work Barrows made some preliminary experiments to determine substances which called forth positive reactions. These experiments consisted of setting up a trap, containing the odorous substance to be tested, in a glass cylinder closed by a glass plate. The air in the cylinder was kept moist by the evaporation of distilled water. A hundred flies which had been previously starved for 24 hours were put into the cylinder, and at the end of another 24 hours the number caught in the trap was recorded. The trap used by Barrows consisted of a vial through the cork of which extended a piece of glass tubing (Fig. 1*b*). Following these preliminary experiments, Barrows used an olfactometer, in which the flies, while passing from a vessel in response to light, were given a "choice" of entering a trap on one side containing odor or a trap on the other side containing water.

Barrows (p. 536) sums up his results as follows:

> These flies are positively chemotropic to amyl and especially ethyl alcohol, acetic and lactic acids and acetic ether.
>
> Acetic ether, isobutyl acetate and methyl acetate, when added in small amounts to 10 per cent ethyl alcohol, greatly increase its attractiveness. A similar increase is noted where acetic or butyric acids are added to the alcohol. All these organic substances are found in fermenting fruits.
>
> The optimum strengths of ethyl alcohol and acetic acid as determined by the number of positive reactions given to different strengths is 20 and 5 per cent, respectively, while a mixture containing 2½ per cent alcohol and ⅝ per cent acetic acid gives a slightly higher number of positive reactions than is given by either 5 per cent acetic acid or 20 per cent ethyl alcohol.

The possibility of improving the methods used in Barrows' preliminary experiments and of more accurately measuring the reactions of *Drosophila* to the products of fermenting banana led to the present experiments.

METHODS

Effect of trap form on catch.—The best trap is obviously one which will catch and retain the greatest number of flies. Trap form is thus a matter of prime importance. Various types of traps tried by previous workers in this laboratory are illustrated in Figure 1. No one of these proved satisfactory. The form of trap finally chosen may be seen in the experimental setup (Fig. 2). It is made from a piece of glass tubing 8½ cm. long and 28 mm. in internal diameter, one end of which has been drawn out and bent in to form an inverted funnel-shaped opening. The lower end of the funnel opening is 2.1 mm. in diameter. The other end of the tube is closed by a cork. In setting up the trap, the odor solution is put into a small glass dish or thimble and set on the cork, and the trap is placed over it.

---

[1] The author wishes to express her appreciation to Dr. D. E. Minnich, her adviser, not only for the suggestion of the problem but also for his invaluable criticism and his inspiring enthusiasm; to acknowledge her gratitude to Dr. C. P. Oliver, who taught her how to culture *D. melanogaster;* to Dr. A. C. Hodson, who has given her some helpful suggestions; and to Dr. Joseph Valasek, who measured the range of the red lamp which was used. She wishes to thank Dr. R. N. Danielson and Dr. Elizabeth Brown Chase for valuable suggestions and German translations, and she wishes also to express her indebtedness to other students who have worked on this problem, among whom are Gertrude Fink McIntosh, Edith Quamme Meincke, Philip Le Compte, and Patty Hynes Wallace.

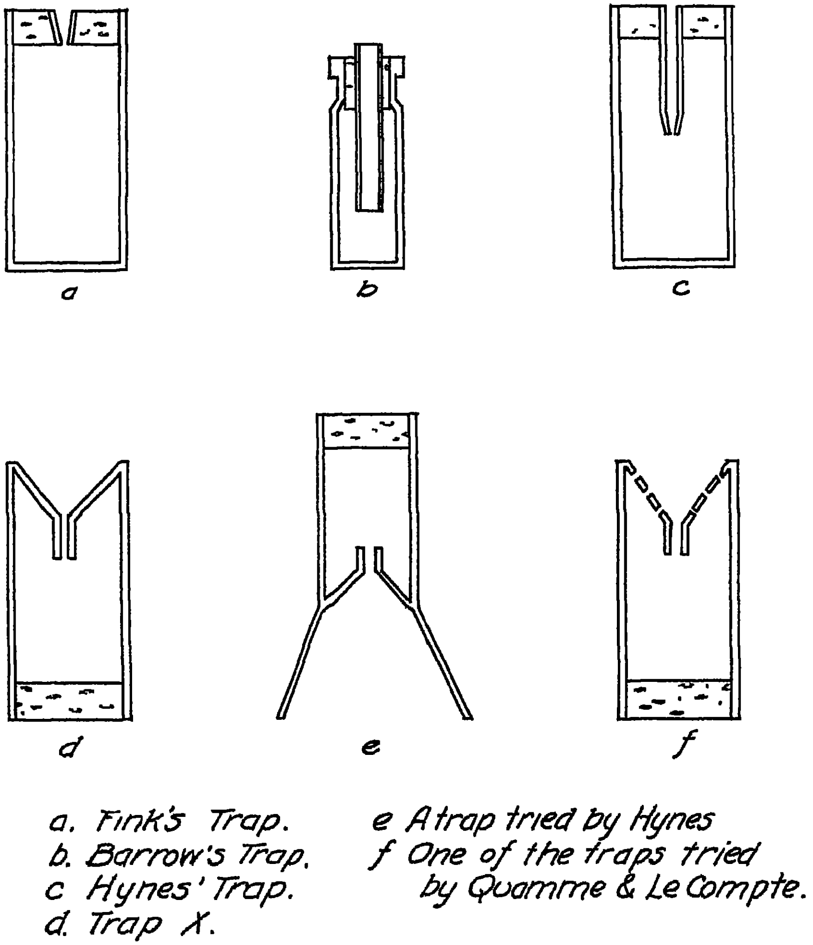


FIG 1.—Traps used by other workers

With a bait of fermenting banana the foregoing trap caught about 90 flies out of 100 in 12 hours, other pertinent factors being under control. A number of such traps were made, and an identification mark scratched on each. In eight experiments 7,113 flies were caught out of 7,755, or 91.7 per cent. Nine out of the eleven traps used caught over 90 per cent, while the two remaining traps caught 87.9 per cent each. The variation in catch ranged from 90 to 98 per cent in the best trap and from 65 to 99 per cent in the poorest trap. The high percentage of flies caught and retained in the traps over a period of 12 hours signified that the escape was small.

The greatest difficulty in the selection of a trap was the variability in catch irrespective of the type of trap employed. This variability led to the investigation of other factors which might be important in influencing the number of flies caught.

Effect of other factors on catch.—Temperature is important for several reasons. The rate at which the fly moves decreases as temperature decreases. Fewer flies are, therefore, caught in the traps at lower temperatures. The fly may make some internal physiological adjustment to the temperature of its environment, so that it may make a difference if the fly is experimented upon at the same temperature at which it is cultured or at other temperatures. This is a question for experimentation.

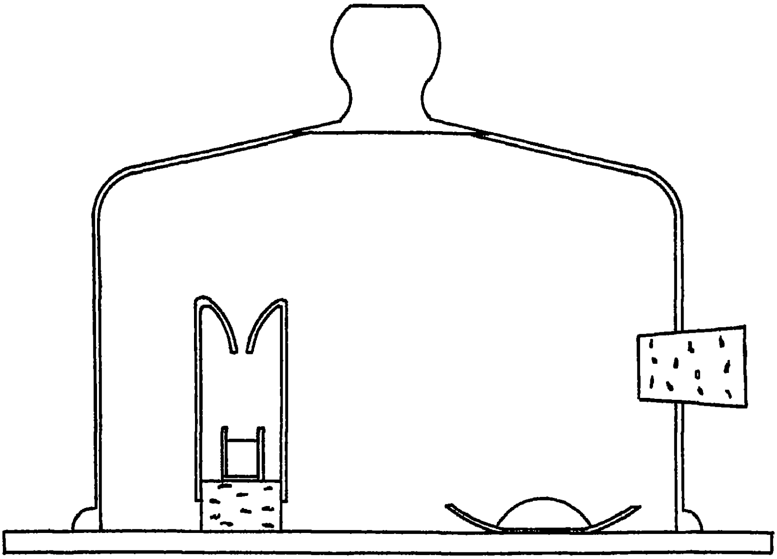


FIG. 2—The experimental setup, consisting of a bell jai on a sand-blasted glass plate, containing a trap with the odor solution and a watch glass of cotton soaked with distilled water to regulate humidity. The hole in the side of the bell jar is used at the beginning of the experiment to insert the counter and at the end of the experiment for etherization of the flies.

The temperature at which flies are cultured is also important in determining the size of the flies; and when the flies are small, the number caught is less than when they are large. Much recent work has been done on this question of relation of temperature to size. Alpatov (1929) found that *Drosophila* reared at 18° C. was larger in a series of bodily dimensions than when reared at 28° C. His method of determining size consisted of taking nine measurements on different parts of each individual. Hersh (1924) and Baron (1935) have studied the effects of temperature on the facet number in *D. melanogaster;* and Stanley (1935) has studied the effect of temperature on wing length, with similar results.

The writer used the average weight per hundred to determine the relative size of flies raised at different temperatures. Since the flies rapidly lost weight because of the evaporation of water from their bodies after etherization, it was necessary to weigh them before counting them. With flies weighing 113.2 mg. per hundred, 90.4 per cent of 1,007 were caught; while with flies weighing 87.7 mg. per hundred, only 62 per cent of 1,008 were caught.

There are three possible reasons why fewer small flies are caught: first, the smaller fly, after entering the trap, may escape more easily than the larger fly; second, the smaller fly may be less active, so that fewer enter the trap; and third, the smaller fly may be less responsive to odors. To test these possibilities the following experiments were performed.

To test the possibility of escape, a hundred flies, weighing about 41.1 mg. per hundred, were counted into each of three traps. Each trap was set under a bell jar, and a dish of fermenting banana was also set under each bell jar. At the end of 2 hours it was found that an average of 36.3 per cent of the small flies had escaped, while an average of 6.2 per cent of the large had escaped. This shows that a considerable number of flies do escape and that it is easier for the small flies to escape than for those of larger size.

In a second experiment ten traps each containing 100 flies (which had been raised at 21° C.) were set as in the previous experiment. After 12 hours it was found that the average percentage of escape for the males was 5.6, while that for the females was 2.4. Since females in *Drosophila* are larger than males, this difference may be due to size.

These experiments thus show that the flies can and do escape when there is an attractive odor outside the trap. The percentage of escape, however, is small when large flies are used.

The effect of activity on catch was tested as follows. Since the visible spectrum of some of the insects is shifted toward the ultra-violet, it seemed possible to find a red light by which the experimenter could observe the actions of the flies under conditions which approach darkness for the flies.[4] The wattage of the lamp chosen was 25; and its range was from 617 $\mu\mu$ to 758 $\mu\mu$, with the maximum brightness at 664 $\mu\mu$. According to Bertholf (1932), the extent of the spectrum for *D. melanogaster* is from 235 $\mu\mu$ to about 650 $\mu\mu$. The stimulating efficiency, however, of the longer wave-lengths is low, the relative stimulating efficiency at 600 $\mu\mu$ being 0.7; at 625 $\mu\mu$, 0.1; and beyond 625 $\mu\mu$ unmeasured.

The red electric light was put into a box (8 × 6 × 4 inches) which was open at the top. Upon the box was placed a sanded glass plate upon which sat a bell jar containing 200 flies. After the flies had been starved for 10 hours, the trap containing the odor was slipped up under the bell jar and all the lights were turned out except the red light. The entrance of the flies into the trap was observed for 15 minutes. During this time no flies were observed to escape from the trap in any of the trials. The factor of escape, therefore, did not enter into these experiments. At the end of the 15 minutes the flies were etherized and counted, and the percentages of males and females were recorded.

In the first set of trials no bait was used in the traps. The flies were divided into four groups of 200 each, weighing 40 mg., 74.3 mg., 112.8 mg., and 117 mg. per hundred, respectively. Each group was placed under a bell jar. More large flies were caught than small flies, and more females than males. The percentage of females caught in each of

---

During the time of trial the flies were kept in darkness.

the four groups was 6.4, 16.0, 32.4, and 36.4, respectively; and the corresponding percentage of males caught was 1.1, 5.6, 16.6, and 20.6.

In the second set of trials the flies were all of the same weight, 117 mg. per hundred. The following baits were used: fermenting banana, 10 per cent acetic acid, 15 per cent ethyl alcohol, and 0.01 per cent amyl acetate. The fermenting banana caught 28.0 per cent females and 4.6 per cent males, the acetic acid caught 0 per cent females and 1 per cent males, the ethyl alcohol caught 34.9 per cent females and 10.6 per cent males, and the amyl acetate caught 26.2 per cent females and 14.2 per cent males.

It is interesting to note that, when a *Drosophila* entered an empty trap, it crept right in without hesitating at the opening; but when it entered an odor trap, it hesitated at this point. With the 10 per cent acetic acid, which acted decidedly as a repellent, the flies, on reaching the opening, either backed out or turned and crept out. The one male which entered flew in directly from the air.

Since no flies escaped, the red-light experiments with empty traps clearly show that there is a difference in activity. The small, undernourished flies raised at high temperatures in crowded conditions are less energetic and active than larger flies raised under optimum conditions. The fact that more females were caught than males must, at least in part, be due to a greater activity on the part of the female or, perhaps better, to a difference in the kind of activity in the males and females. Since females are searching for a place to lay their eggs, they may enter traps oftener than males.

The third point raised above, viz., the relation between the size of the fly and the response to odor, was not determined.

Elimination of anesthetic before trials.—Barrows (1907, p. 531) states: "It should be noted that the process of etherizing has no lasting effect on the ability of the flies to scent food, i.e., normal flies after recovery from ether find food with as great certainty as they did before etherization." He says nothing, however, about the intensity of the etherization or about the time necessary for recovery. The writer has found that under certain conditions etherization has an effect upon the olfactory sense for at least 12 hours after etherization, and perhaps the injury may be permanent.

It was decided, therefore, to use only unetherized flies,[3] which necessitated a special technique. To count the flies without etherizing them, a counter, similar to the one Pearl (1928) used for population counts, was made. The counter (Fig. 3) is made from a piece of glass tubing about 24 cm. long and with an internal diameter of 21 mm. One end is drawn to a funnel opening, 1.8 mm. in diameter and just large enough to let one fly go through at a time; while the other end is flared to fit the top

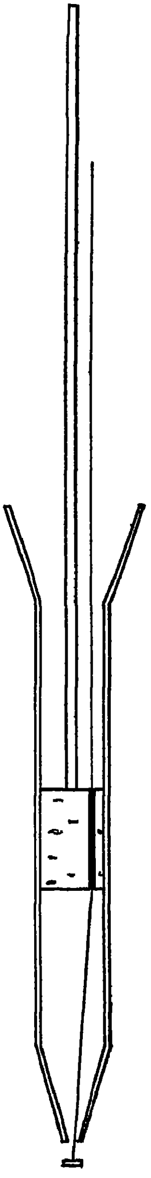


FIG. 3.—The counter used to count flies without etherization.

---

[3] The etherizer consisted of a half-pint milk bottle into the top of which was put an aluminum funnel. The upper edge of the funnel was bent to fit around the top of the bottle; and the lower edge of the funnel tube was slit up and bent back to hold some cotton, which was soaked with ether each time the etherizer was used.

of a pint milk bottle. The plunger, which can be drawn up and down in the tube, is made of cork with a brass rod for a handle. The little valve which closes the opening is a cardboard washer to which is glued a long piece of piano wire. The wire extends from the valve up through the tube and through a small hole burned through the cork to about 4 cm. beyond the tube. By means of this wire the valve may be opened or closed to regulate the number of flies which go through. As a result of using unetherized flies, larger and more constant catches were obtained.

Cultures.—The standard procedure was followed in making the yeast food. The following proportions were used for 20 bottles:

250 cc. bananas
125 cc. white Karo syrup
625 cc water
10 gm agar
½ cc 10 per cent formalin
10 yeast cakes dissolved in water
100 cc. water (added after the agar solution boils)

Twenty flies were counted into each culture bottle (pint milk bottle) without etherizing, since etherizing retards the laying of eggs and the bottles are more apt to become moldy. Most of the flies used in these experiments were raised at 20° C. Flies raised on yeast food and at this temperature are of good size, weighing about 117 mg. per hundred.

General procedure.—The experiments were carried on and the cultures were developed in a small inside room which was about 11 feet long, 8 feet wide, and 11 feet high, maintained at a constant temperature of 20° C. The glassware was washed, the cultures were set, and the odor solutions were made in a near-by room.

The glassware was washed in tap water, rinsed in cleaning solution, tap water, and distilled water, and air dried. The small pieces and the milk bottles were dried in an electric oven, and the bell jars and the glass plates were dried with an electric fan.

For steady experimentation a culture of 20 bottles was set every other day. When the young began to pupate, the parent flies were removed from the culture bottles. Three days after hatching started, the flies were emptied from culture bottles into 2 food bottles by means of an aluminum funnel. The food bottles were quart milk bottles into which about 4 tablespoonfuls of yeast food had set. A half paper towel was put into each bottle to take care of the moisture, and the top was closed with a plug of cotton. The following day some of the flies were emptied into the counter, to be used in experiments. These flies were from 1 to 4 days old. On the second day flies from the same food bottles were used. These were from 2 to 5 days old. No significant difference in reaction was found between the 1–4-day flies and the 2–5-day flies.

In setting up an experiment, 12 bell jars were set on sand-blasted glass plates (Fig. 2) along the edge of a table in the constant-temperature room. A watch glass containing a piece of cotton soaked in distilled water was put under each bell jar, and a fresh cork was put into the hole on the side. A hundred flies were then counted into each bell jar, as previously described; the light was turned off; and the flies were left without food but with water for 10 hours.[4] At the end of this time the light was turned on, and an odor trap was slipped up under each bell jar by pulling the bell jar a little over the edge of the table. The flies were again left in the dark, this time for 12 hours,[4] after which they were etherized by pushing a plug of ether-soaked cotton through the hole in the side of the bell jar and replacing the cork. A count was then made, and the percentage of females and males caught was recorded. The constant-temperature room was then made ready for the next experiment by the removal of the glassware and by airing.

---

[4] The period used by Barrows was 24 hours.

It is well known that *D. melanogaster* is positively phototropic to a high degree. This factor was made use of in these experiments, not only to attract the flies to the top of the bell jar while the trap was being put in, but also to attract the flies through the opening of the counter. The end of the counter was put into a hole which had been bored into the side of the bell jar, and was turned toward the light. The rate at which the flies moved appeared to depend upon the intensity of the light and also upon other factors, such as the amount of moisture in the tube of the counter (moisture collects upon the sides of the tube of the counter if the flies are left in it for any length of time) and also upon the state of general excitement of the flies. This state could be brought about by emptying the flies from the food bottles into the counter. In regard to this state of general excitement, McEwen (1918) says that this fly is not phototropic unless it is in a certain physiological state brought on by, or at least accompanied by, activity.

EXPERIMENTS

It is well known that *Drosophila* is strongly positive to fermenting banana. The question naturally arises as to just which of the products of fermentation calls forth a positive response and in what proportions. Von Loesecke (1929) has studied such fermentations, and the following data are taken from his work. Banana mash contains 16 per cent fermentable sugars. With fermentation the ethyl alcohol content varies from 6.55 to 10.12 per cent. Acetic fermentation gives 5.72 per cent acetic acid. The reactions of *Drosophila* to ethyl alcohol solutions varying from 5 to 30 per cent and to acetic acid solutions varying from 0.1 to 5 per cent were accordingly studied.[5]

Ethyl alcohol.—Ten experiments were made, in each of which 100 flies were tested with each concentration of alcohol and with each of three controls, viz. an empty trap, a trap containing distilled water, and a trap containing fermenting banana.

The results of the experiments are given in Table 1. It is interesting to note that more flies were caught in empty traps than in water traps. Distilled water alone, therefore, acted as a repellent under the conditions of the experiment.

The positive response of females, as shown by the number of individuals responding, increased with an increase of concentration up to between 10 and 15 per cent, beyond which point the positive response decreased with an increase in concentration. Of the solutions tried, the lowest, a 5 per cent solution, called forth a greater positive response among the males than any other solution. With an increase in concentration the positive response of the males decreased. A definite negative response was shown by both males and females to solutions above 25 per cent, since the percentage caught with solutions above this concentration was less than with empty or water traps.

The most plausible interpretation of the difference in response between males and females is that it is a difference in response to odors and not entirely a difference in activity. If the difference in the reactions of males and females to any bait were due entirely to a difference in activity, we would expect this difference to remain constant, irrespective of the bait used. That it does not remain constant shows that there is a difference in reaction to the odor of the bait.

Experiments with acetic acid.—The methods used in the study of the reactions of

---

[5] Solutions were made by volume.

Drosophila to acetic acid were the same as those used with ethyl alcohol solutions. The results of eight experiments, using 800 flies for each concentration, are given in Table 2. Solutions of 0.1, 0.2, 0.4, 0.6, 1, and 5 per cent concentrations were used. The writer's results differ greatly from those of Barrows (1907). He found the optimum strength of acetic acid, as determined by the number of positive reactions given to different strengths, to be 5 per cent (including the responses of both males and females). The writer found 0.4 per cent to be optimum for females and 0.2 per cent to be optimum for males, while a solution of 5 per cent or greater (tried in preliminary experiments) acted as a repellent. A 0.4 per cent solution caught 59.1 per cent of females, and a 0.2 per cent solution caught 40.9 per cent of males. A 5 per cent solution caught 3.9 per cent females and 0.8 per cent males.

TABLE 1

SUMMARY OF TEN EXPERIMENTS, SHOWING CONTROLS AND RESPONSE TO ETHYL ALCOHOL

Period of starvation, 10 hours. Time of trial, 12 hours. Age of flies, 1–4 and 2–5 days. Temperature, 20° C.

BAIT	NO. OF FLIES CAUGHT IN TRAP		NO. OF FLIES NOT CAUGHT		PERCENTAGE CAUGHT	
	Female	Male	Female	Male	Female	Male
Empty	107	149	252	506	29.8	22.7
Water	51	65	296	594	14.7	9.9
Banana	444	379	24	157	94.9	70.7
5 per cent alcohol	274	211	189	321	59.2	39.7
10 per cent alcohol	336	163	185	324	64.5	33.5
15 per cent alcohol	259	199	146	380	63.9	34.4
20 per cent alcohol	239	72	353	355	40.4	16.9
25 per cent alcohol	89	32	434	437	13.2	6.8
30 per cent alcohol	34	17	454	510	7.0	3.2

TABLE 2

SUMMARY OF EIGHT EXPERIMENTS, SHOWING CONTROLS AND RESPONSE TO ACETIC ACID

Period of starvation, 10 hours. Time of trial, 12 hours. Age of flies, 1–4 and 2–5 days. Temperature, 20° C.

BAIT	NO. OF FLIES CAUGHT IN TRAP		NO. OF FLIES NOT CAUGHT		PERCENTAGE CAUGHT	
	Females	Males	Females	Males	Females	Males
Water	54	81	233	441	18.8	15.5
Banana	252	436	8	116	96.9	78.9
0.1 per cent acid	151	64	240	334	38.6	16.1
0.2 per cent acid	196	159	220	229	47.1	40.9
0.4 per cent acid	279	97	193	229	59.1	29.7
0.6 per cent acid	236	107	198	281	54.4	27.6
1 per cent acid	115	83	316	288	26.7	22.4
5 per cent acid	16	3	396	396	3.9	.8

In regard to the repellent action of acetic acid, it was observed during the experiment with the red light that with a 10 per cent solution as bait the flies crawled down the funnel of the trap to the opening and then either backed out or turned and crawled out. Some stopped part way up the funnel to clean the head and abdomen.

SUMMARY

A series of experiments to determine the olfactory reactions of *D. melanogaster* to solutions of various concentrations of ethyl alcohol and acetic acid is reported, with the following results.

1. A trap has been perfected which will catch about 90 flies out of 100 in 12 hours when fermenting banana is used as bait.
2. A new valve has been invented for Pearl's counter which makes it possible to count out a definite number of flies at a time. This makes it unnecessary to etherize flies in order to count them, and thus does away with the possibility of the detrimental effects of etherization upon the olfactory sense.
3. A method of observing the activity of *Drosophila* under conditions which approach darkness for the fly has been described.
4. Large flies are more active than small flies.
5. Females are more active than males, as indicated by the fact that more females are caught in empty traps than males.
6. There is a difference between males and females in their response to odors.
7. Excessive moisture acts as a repellent, as is shown by the fact that empty traps catch more flies than water traps.
8. *Drosophila* females are strongly positive to solutions of ethyl alcohol up to 25 per cent, with a maximum response at 10–15 per cent; and *Drosophila* males are definitely positive to ethyl alcohol solutions up to 15 per cent, with a maximum response at or below 5 per cent.
9. *Drosophila* females are strongly positive to acetic acid in concentrations up to 1 per cent, with a maximum response at 0.4 per cent; and *Drosophila* males are positive to acetic acid in concentrations up to 1 per cent, with a maximum response at 0.2 per cent.
10. Solutions of ethyl alcohol above 25 per cent and of acetic acid above 5 per cent act as repellents to both males and females.

LITERATURE CITED

ALPATOV, W. W. 1929. Influence of temperature on *Drosophila melanogaster*. Amer. Nat., 63:37.

BARON, ABRAHAM L. 1935. Facet number in *Drosophila melanogaster* as influenced by certain genetic and environmental factors. Jour. Exper. Zoöl., 70:461.

BARROWS, WILLIAM MORTON. 1907. Reactions of the pomace fly to odorous substances. Jour. Exper. Zoöl., 4:515.

BERTHOLF, L. M. 1932. The extent of the spectrum for *Drosophila* and the distribution of stimulative efficiency in it. Zeitschr. f. wiss. Biol., Abt. C, Zeitschr. f. vergl. Physiol., 18:32.

HERSH, A. H. 1924. The effects of temperature upon the heterozygotes in the bar series of *Drosophila*. Jour. Exper. Zoöl., 39:55.

LOESECKE, HARRY VON. 1929. Preparation of banana vinegar. Ind. and Eng. Chem., 21:175.

MCEWEN, R. S. 1918. The reactions to light and gravity in *Drosophila* and its mutants. Jour. Exper. Zoöl., 25:49.

PEARL, RAYMOND. 1928. The rate of living. New York: Knopf.

STANLEY, WILLARD F. 1935. The effects of temperature upon the wing size in *Drosophila*. Jour. Exper. Zoöl., 69:459.

RACE AND CONJUGATION OF PARAMECIUM

ARTHUR C. GIESE
School of Biological Sciences, Stanford University

MANY investigators, among whom the most recent are Sonneborn and Cohen (1936), have reported that some races of *Paramecium* and of other protozoa conjugate readily while other races of the same species conjugate rarely, if at all, under apparently identical conditions. It is possible that different races respond to variations in different environmental factors, for changes in each of the following factors: (1) food supply (Maupas, 1889, and many others), (2) metabolites (Chatton and Chatton, 1931), (3) pH (Austin, 1927), (4) salt type and concentration of the medium (Enriques, 1909, and Zweibaum, 1912), (5) population density (Mast, 1917), (6) oxygen tension (Enriques, 1909), (7) temperature (Prandtl, 1906), and (8) the type of substrate (Baitsell, 1912) in which the organisms are grown, have been invoked but not conclusively proved as possible factors which, singly or concomitantly with other factors, induce conjugation in conjugating races of diverse protozoans.

In a recent study of a conjugating race of *Paramecium multimicronucleatum* Powers and Mitchell, when most of the other factors were kept relatively constant, conjugation was found to be closely correlated with a decrease in available food after a period of plenty (Giese, 1935) when accompanied by a simultaneous change in temperature from 26° to 20° C. The change in temperature alone was ineffective in inducing conjugation. It seemed interesting (1) to compare the conditions necessary for induction of conjugation in a number of other races of the same species of *Paramecium*, and (2) to determine, should any of the other races fail to conjugate under the conditions which are correlated with conjugation in the race already studied, whether changes in any of the other environmental factors listed above might induce conjugation.

MATERIALS AND METHODS

The races of *Paramecium*, which for convenience have been numbered 1, 2, 6, 7, 8, 9, and 11, were all isolated in the vicinity of Stanford University but in different habitats and at widely separated localities. Race 1 was isolated in the fall of 1932; race 11 was obtained from this race in 1935; all the others were isolated in the spring of 1936.

The culture methods were similar in most respects to those already reported. Briefly, the paramecia were grown on a single strain of bacteria (*Pseudomonas ovalis*) in buffered (pH 7.0) lettuce extract at 26 ± 0.1° C., one animal being inoculated per cubic centimeter of medium, and were prevented from conjugation by subculturing until conjugation tests were made ($3\frac{1}{2}$ days after inoculation). The main change in culture method from that previously reported was filtration of the medium, instead of decanting, to eliminate the particles of lettuce which interfered with the conjugation tests. Preliminary tests showed that medium filtered through ordinary qualitative filter paper was unfit for use; thus, whereas in controls there were, on the average, 284 animals per cubic centimeter 3 days following subculture, in filtered medium there were but 5 per cubic centimeter. Medium filtered through Seitz and Chamberlain bacteriological filters was so toxic that the animals rarely divided, became bloated and burst, dying long before the death of controls starving in balanced inorganic salt solution. It was later found that the qualitative paper could be rendered innocuous by a wash with hot lettuce medium (not, however, by hot distilled water). Furthermore, quantitative paper was generally nontoxic, but filtration was slow and tedious. Coarse, gray filter paper did not seem to introduce any toxic materials and was far more convenient than quantitative paper; therefore, following a preliminary "safety" wash with hot culture fluid, it was used for filtering the culture media employed in these experiments.

The method used for inducing conjugation was essentially that previously described (Giese, 1935). Animals from cultures in which multiplication had practically ceased were concentrated twenty fold by centrifugation and placed in watch glasses at 20° C. While concentration is unnecessary, it results in a more rapid reduction of remaining food. A change in temperature was found to be unnecessary for some of the races; but since the epidemic lasts longer at the lower temperature, such a change facilitates observations. When the effect of a given solution was to be studied, the paramecia were washed and suspended in that solution in the manner previously described.

The isolation culture method used to determine viability of exconjugants of the different races studied was as follows. A single conjugant pair was placed in a tube 5 mm. by 7 cm., containing about $\frac{3}{4}$ cc. of the lettuce medium described, which was diluted half and half with distilled water, a very light inoculation with *P. ovalis* being made at the same time. Observations were then made at 12-hour intervals. Previous studies had shown that higher concentration of culture medium is unfavorable, since multiplication of exconjugants is too slow to keep apace of bacterial development.

Dishes and glassware were carefully cleaned and sterilized by autoclaving or dry sterilization. Bacteriological precautions were observed for all organic solutions and cultures in such solutions; inorganic solutions were sterilized to prevent entrance of extraneous protozoans. Most of the experiments were performed in Columbia watch glasses or in 1-inch Syracuse watch glasses; some in 3-inch watch crystals. All were appropriately moist-chambered.

The inorganic balanced salt solution referred to in the paper is a modified form of the solution used by Barker and Taylor (1931), consisting of 6 per cent solutions of the following salts mixed in the proportions given: NaCl, 1,000 parts; $MgCl_2$, 78 parts; $MgSO_4$, 38 parts; KCl, 22 parts; $CaCl_2$, 10 parts. This mixture was diluted five hundred times and buffered at pH 7.0 with 1 part stock buffer added to 9 parts salt solution. The stock buffer was made by titrating 50 cc. of a 0.20 M KH_2PO_4 solution to pH 7.0 with 0.2 M NaOH and making up to 200 cc. All solutions were made up with water redistilled in an all-pyrex still.

In all studies reported, unless otherwise stated, at least ten experiments were performed on at least three different days to obtain evidence on a given point. When experiments were negative, usually thirty experiments were performed, and sometimes even more. Measurements of animals reported are averages obtained from twenty-five individuals. For such measurements a camera lucida and a stage micrometer were used. The animals to be measured were quickly injected into Dafano's fixative or methyl green and outlined under camera lucida (without pressure from a cover slip). For permanent mounts the animals were fixed in Schaudinn's fixative and stained with Feulgen's nuclear stain, except those of race 1, which were stained with iron hematoxylin.

EXPERIMENTAL

1. *The races of Paramecium used in the investigation.*—Each of the races selected for this study showed characteristics by which it could be distinguished from the others. Members of race 11 were least vigorous, dividing, on the average, but 1.42 times per day; members of race 8 were somewhat slower than the remainder, with a division rate of 2.31 per day; members of races 2, 7, and 9 showed a division rate of about 2.5 per day; and representatives of race 6, 2.71 per day. The data, each from twenty counts and averages for 3-day periods (5 days for race 11), of the number per cubic centimeter in mass cultures are given in Table 1.

All races save 1 and 11 were able to thrive on a suspension of *P. ovalis* in inorganic salt solution as well as in nutrient solution, ten successive transfers being tried in each case. Of the races tested (1, 2, 6, and 11), only members of race 2 were able to thrive on either *Bacillus subtilis* or *Pseudomonas fluorescens* in lettuce; members of race 6 grew luxuriantly on *P. fluorescens* for a time, then died out; members of race 11 grew for a period of three transfers on each of the foregoing bacteria.

TABLE 1

COMPARISON OF THE RACES OF *Paramecium* STUDIED

Race No.	Length in μ	Width in μ	Division Rate per 24 Hours	Percentage Showing Conjugants in Consecutive Trials*	Percentage of Conjugants Viable†
1..............	183.3±24.2	56.5±8.8	2.47	97.0 (66)	Low
2..............	187±20.2	59.3±2.3	2.50	88.6 (26)	50 (20)
6..............	189±15.3	69±7.2	2.71	85.2 (27)	{10 (40) 68 (60)
7..............	199±21.8	77±4.8	2.42	96.4 (28)	25 (40)
8..............	203±14.6	66±8.2	2.31	84.2 (19)	65 (20)
9..............	162±26.1	64±4.7	2.54	100.0 (27)	48 (35)
11..............	171±20.0	49.4±6.0	1.42	100.0 (25)	5.6 (67)

* The numbers in parentheses indicate the number of consecutive trials made.

† The numbers in parentheses are the total number of trials made.

Individuals of race 9 were shortest of all, being, on the average, 162 × 64 μ; those of race 11 were not much larger, being 171 × 49 μ; those of race 8 were the longest, being 203 × 66 μ; all of the others were of more or less the same size, individuals of race 7 being a bit more rotund than the others. Members of race 2 were generally filled with many more excretion crystals than any of the others and were, in general, more active even during conjugation.

Paramecia of all the races save 11 show micronuclei clearly; but the number is variable, a finding established as the general rule for this species of *Paramecium* by the careful work of Stranghöner (1932). Generally four or fewer micronuclei were seen; in many individuals only one could be seen; in some individuals of race 6 no micronuclei could be observed, although others from the same culture showed them clearly. Such apparently amicronucleate individuals were also observed by Powers and Mitchell in their stocks (1910). In hundreds of specimens of race 11 examined in all stages—dividing, conjugating, in the normal vegetative stages, as well as in exconjugants—no micronuclei could be observed following the Feulgen technique originally used. But when the time in acid was increased to about 20 minutes and the time in fuchsin sulphurous solution to 24 hours, the micronuclei were plainly visible in all the specimens observed. The macronuclei of some members of race 11 showed fragmentation, a phenomenon occasionally observed in specimens of race 6.

Exconjugants of the different races exhibited variable viability. Races 1, 7, and 11 showed rather low viability, only 25 per cent of the exconjugants of race 7 giving rise to good cultures, and only 5.6 per cent of the exconjugants of race 11. Exconjugants of race 6 showed variable viability. In one series of tests only 10 per cent survived; in another, 68 per cent. Exconjugants of races 8 and 9 showed high viability, 65 and 48 per cent, respectively, in the tests made.

2. *Conjugation tests.*—After subculturing had been carried on for several months, conjugation tests were made. For all of the races except race 2, conditions similar to those already described for race 1 were found to be correlated with conjugation. Twenty to thirty tests were made on consecutive days, and other tests were made later at various times. All cases except in trials on races 9 and 11 gave a few negative results. Thus, in 3 per cent of the trials with race 1, in 14.8 per cent with race 6, in 3.6 per cent with race 7, and in 15.8 per cent with race 8 no conjugants were obtained. However, in some cases the conditions were unfavorable for unknown reasons, and death of a few or of many animals occurred; in other cases the cultures were apparently entirely normal, yet no conjugation occurred. Also, on a variable percentage of days the epidemics in cultures of some races were very weak, only 5–10 per cent being in conjugation; for race 8 in 36.8 per cent of the trials such small epidemics were obtained, for other races the percentage was much lower. Sonneborn (1936) has found a correlation between time since previous nuclear reorganization and conjugation. No evidence indicating a rhythm was here obtained, but the study did not extend over a sufficient period of time. A further study is being made. However, when normal epidemics occurred, they were closely correlated with the depletion of food. At least ten trials in which the animals were washed free of nutrient medium with buffered balanced inorganic salt solution were also made with each clone and the epidemics of conjugation were similar to those in the unwashed controls.

If the paramecia are fed a suspension of *P. ovalis* at the time they are concentrated, the epidemic of conjugation is effectively delayed, the delay depending on the amount of food given. A 1-mm. loopful of a 24-hour growth of *P. ovalis* grown on 2 per cent yeast extract in 2 per cent agar (26° C.), scraped even with the edges, was suspended in 1 cc. of buffered balanced salt solution to make a "standard" suspension. Two drops of such a suspension for the animals from 7½ cc. of a standard culture (third day) did not delay conjugation for more than a few hours; 5 drops, however, produced an easily measurable delay of from 12 to 24 hours, depending upon the race and culture. Ten trials were made with race 6, three for each of the others. Therefore, as in the experiments on race 1 previously reported, here, too, feeding delays the onset of the epidemic of conjugation.

Race 2, tested in the same manner, was the single exception. This race was grown in the standard manner for over 5 months before tests were begun. No conjugants were observed over this entire period. Sixty tests were then made in the standard manner over a period of about a month; all were negative. Examinations at 12-hour intervals were made for 3 days on most cases, and in some cases for a week, to make certain that the epidemic of conjugation was not missed. A sticky state appeared at the time conjugation would have occurred in a conjugating race, but not a single union was observed.

Therefore, experiments were now performed in which each of a number of other factors were varied concomitantly with the change in food supply. Thirty trials were made in each case. Changes in (1) substrate from lettuce (*a*) to 0.1 per cent oat-hay mixture and (*b*) to 0.05 per cent *Agaricus;* (2) pH from 7.0 (*a*) to 6.0 and (*b*) to 8.0, using comparable buffered inorganic salt solutions; (3) salt medium (*a*) to M/60,000 $FeCl_3$ and (*b*) to M/48,000 $AlCl_3$, both of which were found effective incitants by Zweibaum (1912); (4) bacteria used as food from *P. ovalis* (*a*) to *P. fluorescens* and (*b*) to *B. subtilis*, food bacteria being considered important by Chatton and Chatton (1931), were all without effect, for no conjugants appeared. Since observation lasted over 3 days after setting out a given test, it is unlikely that even chance conjugants would have been overlooked.

Jennings (1910) reports successive starvation as effective in *Paramecium aurelia* for induction of conjugation. A series of two and three successive starvations was tried without success. Starved animals kept at 26° C. were also observed, but no conjugation occurred. Trials were also made in which animals were taken from cultures before the maximal population had been reached and 12, 24, and 36 hours after the maximal numbers were reached in standard cultures. All proved negative.

Just at the conclusion of the foregoing series of 320 negative experiments, and when the race had been in subculture for over 7 months, conjugants appeared, at first in small numbers and then in large numbers, both in cultures in lettuce-extract fed *P. ovalis* and in cultures fed *B. subtilis.* They appeared at the time the bacterial food was depleted (when the opacity due to suspended bacteria had disappeared). When such cultures were concentrated twenty fold and set out at 20° C., excellent epidemics in which 60–80 per cent were in conjugation appeared. In a series of twenty-six daily consecutive trials, 11.4 per cent showed no conjugation, 34.6 per cent showed rather poor epidemics, and all the others showed excellent epidemics. The reason for failure of a certain percentage to conjugate is not clear; in only a few cases was injury apparent. The interesting point, however, is that conjugation should suddenly appear and occur under exactly the same conditions found to hold for the other races and which previously had failed utterly to evoke even a single conjugant. It seems most likely that some profound change had taken place in the organism which now enabled it to respond to the change in the environmental conditions. The epidemics were observed to occur for the following 7 months, during which the culture was under observation. When the animals were fed a suspension of bacteria, the epidemic was similarly delayed, as in the experiments on the other races already described.

The experiments on race 2 indicate that (1) the proper internal conditions are necessary to secure conjugation; (2) if these are favorable, conjugation will occur following a sudden depletion of the food supply in race 2, just as in the other races tested; and (3) variations in the environmental conditions other than food will not induce conjugation in this race when the internal conditions are such as to be unaffected by the sudden decrease of the food supply.

In the course of observations of epidemics of conjugation in various races over the last 5 years, in only a few instances were almost all the paramecia in conjugation (usually about 60 per cent were united at a given time); at other times a much smaller number or a much larger number were united at the peak of an epidemic. It seemed possible that some internal change had taken place in members of a given clone such that in a given population some of the subraces were "nonconjugating races," others "conjugating races." To test this supposition, thirty individuals of race 6 were isolated and cultures were grown of each. When these cultures were tested for conjugation in the usual way, the individuals in all save one (in which abnormal development had occurred) conjugated, and the epidemics were comparable but not better than those usually found in the parent 6 stock. There therefore seemed to be no separation of nonconjugating races, and the failure of some individuals to conjugate must be explained in some other way.

To determine whether individuals not united at the peak of an epidemic would conjugate later, such individuals were picked out and observed for a period of time. Only occasional unions occurred, and observations of stained specimens obtained 12, 24, 48, and 72 hours after isolation from the culture in which conjugation was occurring indicated that no nuclear changes were occurring in the majority of the individuals. A very few showed macronuclear breakdown; these might have been exconjugants.

The nuclear behavior indicates that in all races we are dealing with true conjugation. Conjugants show the characteristic ribbon formation during macronuclear breakdown. Exconjugants fixed and stained 12–24 hours following isolation of conjugants almost invariably show macronuclear breakdown. Only 2 exconjugants out of 100 in race 6 had their macronuclei intact; but in race 9, 20 out of 88 examined had their macronuclei intact. Exconjugants of all other races examined gave the characteristic macronuclear breakdown.

In conclusion it appears that both internal and external environmental factors influence the induction of conjugation. When the internal state was "unfavorable," conjugation was not induced by variation of a number of environmental factors tried. Sonneborn (1937), in his exceedingly interesting analysis of conjugation of members of certain races of *P. aurelia*, has shown that presence of two sexes in a culture is a necessary condition for conjugation. Preliminary trials with the races used here indicate that, with the possible exception of race 2, there is no clear indication of sexual differentiation. These studies will be reported in full at a later date. However, when conjugation occurred, it was found to be correlated with comparable environmental conditions for each of the seven races investigated.

SUMMARY

1. Seven races of *P. multimicronucleatum* were found to conjugate under similar environmental conditions.
2. The main change in the environment correlated with conjugation was the depletion of food after a period of plenty.
3. Feeding suspensions of bacteria to paramecia effectively delays the epidemics of conjugation, the delay depending upon the supply of food given.
4. One of the races which in the beginning did not conjugate under the foregoing conditions was unresponsive to changes in temperature, substrate, pH, $FeCl_3$, or $AlCl_3$ solutions, and bacterial food upon which grown, when each of these was varied concomitantly with decrease in food following successive starvations.
5. This race suddenly began to conjugate, and the epidemics induced occurred under conditions similar to those obtaining in the other races.

LITERATURE CITED

Austin, M. L. 1927. Studies on *Uroleptus mobilis*. I. An attempt to prolong the life cycle. II. The conditions necessary for conjugation. Jour. Exper. Zoöl., 49:149–216.

BAITSELL, G. A. 1912. Experiments on the reproduction of the hypotrichous Infusoria. I. Conjugation between closely related individuals of *Stylonychia pustulata*. Jour. Exper. Zool., 13:47–78.

BARKER, H. A., and TAYLOR, C. V. 1931. A study of the conditions of encystment of *Colpoda cucullus*. Physiol. Zool., 4:620–34

CHATTON, M. E., and CHATTON, Mme M. 1931. La Conjugaison du *Paramecium caudatum* determinée expérimentalement par modification de la flore bactérienne associée. Race dites conjugantes et non-conjugantes. Compt. rend. Acad. sci., 193:206–8.

ENRIQUES, P. 1909. La conjugazione e il differenziamento sessuale negli Infusori. III. Azione dei sali sulle epidemie di conjugazione nel *Cryptochilum nigricans*. Mem. Roy. acad. sci. Inst. Bologna, Ser. VI, 6:463–500.

GIESE, A. C. 1935. The role of starvation in conjugation of *Paramecium*. Physiol. Zool., 8:116–25.

JENNINGS, H. S. 1910. What conditions induce conjugation in *Paramecium?* Jour. Exper. Zool., 9:279–98.

MAST, S. O. 1917. Conjugation and encystment in *Didinium nasutum* with especial reference to their significance. Jour. Exper. Zoöl., 23:335–59.

MAUPAS, E. 1889. La Rajeûnissement karyogamique chez les ciliés. Arch. zoöl. expér. et generale, 7:149–517.

POWERS, J. H., and MITCHELL, C. 1910. A new species of *Paramecium* (*Paramecium multimicronucleata*) experimentally determined. Biol. Bull., 19:324–31.

PRANDTL, H. 1906. Die Konjugation von *Didinium nasutum* F. M. Archiv f. Prot., 7:229–58.

SONNEBORN, T. M. 1936. Factors determining conjugation in *Paramecium aurelia*. I. The cyclical factor. the recency of nuclear reorganization. Genetics, 21:503–14.

———. 1937. Sex, sex inheritance and sex determination in *Paramecium aurelia*. Proc. Natl. Acad. Sci., 23:378–85.

SONNEBORN, T. M., and COHEN, B. M. 1936. Factors determining conjugation in *Paramecium aurelia*. II. Genetic differences between stocks or races. Genetics, 21:515–18.

STRANGHONER, E. 1932. Teilungsrate und Kernreorganizationsprozess bei *Paramecium multimicronucleatum* Powers and Mitchell. Arch. f. Prot., 78:302–60.

ZWEIBAUM, J. 1912. Les Conditions necessaires et suffisantes pour la conjugaison du *Paramecium caudatum*. Arch. f. Prot., 26:275–393.

REPRODUCTION OF PARAMECIUM IN OLD CULTURE MEDIUM

(Three figures)

W. H. JOHNSON AND GARRETT HARDIN
Stanford University

ALLEE (1931) states that "there is a general ecological assumption that the accumulation of the waste products of a given species in their habitat tends, with most animals, to limit the time of their occupancy, at the same time preparing the way for another species to come in." In his discussion of bacteria Henrici (1928) says: "The idea that growth is limited by the accumulation of some toxic substance is the one that seems to be most generally accepted, though the evidence for it is far from being convincing." The idea contained in these two statements relative to the role of waste products has been restated or assumed in most protozoan studies of the last forty years.

In the first careful work on the subject Woodruff (1911) found an effect of waste products when from 1 to 4 animals had lived in about 0.5 cc. of medium for 24 hours. Using volumes of media, periods of time, and numbers of animals comparable to those employed by Woodruff, Myers (1927), Calkins (1926), Greenleaf (1926), Peterson (1929), Di Tomo (1932), and Beers (1933) obtained depressing effects of excretory products on protozoan division-rates. With reference to bacteria-free cultures of *Glaucoma*, Jahn (1934) says: "The fact that the supply of *N* compounds is not greatly decreased before growth stops indicates that cessation of growth is caused by the accumulation of waste products." Gause (1934) in his extensive experiments with protozoan populations accepts the conclusions of Woodruff concerning the effects of waste products on division-rate and states that he has eliminated the complicating influence of these substances by changing the medium in his cultures every 24 hours.

A few other investigators have reported results which differ considerably from those referred to above. Dimitrowa (1932) found that a very small amount of old culture medium added to fresh medium caused *Paramecium* to divide faster than they did in nonconditioned medium. Phelps (1936) obtained populations of *Glaucoma* grown in pure culture on yeast extract as dense as 500,000 per cubic centimeter. He states that concentrations of animals more than one thousand times greater than those observed in other investigations were obtained before the effects of excretory products on division-rate were noticed. In considering these differences, Phelps made two suggestions. In some cases he considered that the difference might be due to the fact that, as a population of mixed bacteria were growing along with the protozoa, the waste products of these bacteria might have had a depressing effect on the protozoan division-rate. He also suggested that different kinds of food might cause the same protozoan to produce excretory products quite different in nature and effects.

Recently, Taylor and Strickland (1938) have been able to grow *Colpoda duodenaria* in 500-cc. cultures of balanced salt solution with a single kind of bacterium as the source of food, with uniform growth-rates. They have found that excretion products, accumulating in the medium for 4 months, do not affect the growth curves.

In previous studies (Johnson, 1933 and 1937) it was pointed out that experiments were needed to test the relative roles of food and waste products on division-rate in protozoan populations feeding on bacteria. To do this has been the aim of these studies.

MATERIALS AND METHODS

The experimental animals were taken from a clone of *Paramecium multimicronucleata* which has been maintained in this laboratory for some time. The paramecia were grown in a nonnutritive balanced salt solution on suspensions of a strain of the bacterium *Pseudomonas fluorescens* obtained from the Stanford University bacteriology department. The bacteria were grown on agar plates. The method of making the salt solution has been described elsewhere (Johnson, 1933). A platinum loop with an inside diameter of 1.5 mm. was used to measure the suspensions. The paramecia were cultured in 5 and 10 cc. of the liquid with 1 loopful of bacteria for each 5 cc. of liquid as the standard suspension (X concentration). Preliminary experiments using 1 *Paramecium* in 2 drops of different bacterial densities gave results which indicated that the density of bacteria used in the standard suspensions was about the optimum for this ciliate at 20° C. In the 5-cc. cultures, 80 paramecia were introduced at the beginning of the experiment. In the 10-cc. cultures, 160 animals were used.

The culture dishes were kept in moist chambers at constant temperatures. In some experiments the temperature was 20° C.; in others, 26° C.

The cultures were centrifuged for counting. A glass rod with a ball on one end was used as a plunger to trap the paramecia in the bottom of the centrifuge tube. In this manner it was possible to trap all of the paramecia in approximately ½ cc. of the culture fluid. The remainder of the liquid was poured from the tube, and in the case of the experimental cultures was used with ½ cc. of fresh medium for the next day's culture fluid. In every case the liquid poured from the centrifuge tube was examined for paramecia. The control cultures were made up each day from fresh medium. By this method the experimental cultures consisted of 4½ cc. of old medium plus ½ cc. of fresh medium. It was assumed in the beginning that the experimental culture medium would accumulate more and more waste material as it was used over and over again. To take account of any possible effect of the ½-cc. dilution each day, other experiments were devised which will be described later.

For the actual counts the paramecia trapped in the centrifuge tube were transferred to a large depression slide, and the counts were made with a micropipette. The animals for each experiment were taken from the cultures of the preceding day.

To insure the same amount of food in both control and experimental cultures, where ½ cc. of liquid was lost from centrifuging, a dense suspension of bacteria was made by adding 5 loopfuls of bacteria to 2½ cc. of salt solution; and then ½ cc. of this suspension was added to 4½ cc. of culture fluid in each case. In those experiments where no liquid was added to the old culture medium, attempts were made to fill the loop with the same amount of bacteria each time.

Unless stated otherwise, the culture fluid was filtered through a Chamberland-Pasteur porcelain filter. The salt solution was buffered (as previously described) to a pH of 6.9–7.0, and the pH of the culture fluid was tested each time before the fluid was discarded.

Any deviations from the above-described methods will be stated in the discussion of the experiments.

EXPERIMENTAL

At the outset the best approach to the problem seemed to be in the use of the same medium over and over, filtered each day to remove all the bacteria. In this way the control series and the experimental series with the old medium could be kept the same with respect to the amount of food.

TABLE 1

USE OF FILTERED OLD MEDIUM

DAYS	DIVISION-RATES		DAYS	DIVISION-RATES	
	Control	Experimental		Control	Experimental
1	2.04	....	11	1.63	1.29
2	1.96	1.85	12	1.10	1.20
3	2.03	2.17	13	1.25	1.16
4	2.46	2.11	14	2.25	2.15
5	1.91	1.28	15	2.11	1.93
6	1.87	1.34	16	1.76	1.09
7	2.30	2.06	17	2.21	2.29
8	1.28	1.03	18	1.04	1.62
9	1.40	1.83	19	2.07	2.00
10	2.03	1.60	Mean	1.81	1.66

Probability*: Control versus experimental, $P = .0746$

* All calculations by the method of "Student" (1925).

Table 1 shows the results of such an experiment over a 19-day period. The fission-rates given here are the averages of two controls and two experimentals each day. As approximately 1 cc. of fluid was lost in the candle, the media from the two 5-cc. experimental cultures were mixed and filtered together. Each culture lost ½ cc. of medium in centrifuging. Thus, each culture lost altogether 1 cc. of medium. This loss was made up with fresh medium. The standard suspension of bacteria was used, and the cultures were kept at 20° C. New cultures were seeded with animals from the same type culture of the preceding day.

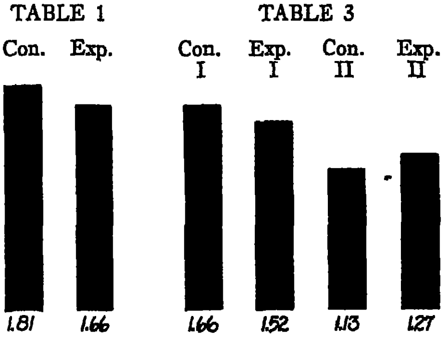


FIG. 1.—Showing the mean fission-rates from Tables 1 and 3.

The mean fission-rate of the controls is slightly higher than the experimentals, but the difference is not statistically significant. On the basis of many of the results cited above, it was anticipated that there would be a gradual decrease in the fission-rate of the experimental series. This, however, was not the case. It seemed that either there was no pronounced effect of waste products for this period of time or the filter removed such substances and prevented their effects. Eijkman (1907) and Curran (1925) found that growth-inhibiting substances from bacterial cultures would not pass through porcelain filters, at least not in the early stages of the filtering process. So it seemed that this sort of thing might have happened here.

In the preliminary experiments using small volumes of the standard suspension (in the ratio of one organism for each drop of medium) the medium appeared absolutely clear after 24 hours. Apparently, the paramecia had eaten most of the bacteria. Because of this it seemed that it might be possible to use the same medium over and over, unfiltered, without producing bacterial crowding of the protozoans.

Before describing such experiments it seems advisable to refer to a difficulty encountered in the use of unfiltered salt solution. Both with control and with experimental cultures it was possible to obtain consistent results for several days, and then the fission-rate would drop and in some cases the cultures would have fewer organisms than the original seeding at the end of 24 hours.

TABLE 2

COMPARING FILTERED AND UNFILTERED MEDIUM AT 26° C.

Days	Filtered	Unfiltered	Days	Filtered	Unfiltered
1	2.02	0.72	15	2.55	2.30
2	1.99	1.60	16	2.22	(62)*
3	2.05	0.95	17	2.16	0.86
4	2.18	1.87	18	1.77	1.09
5	2.34	2.22	19	2.25	2.20
6	2 02	1.82	20	2.24	2.02
7	2.49	2.23	21	2.18	1.50
8	2.16	1.92	22	2.21	(17)*
9	2.07	2.07	23	2.48	1.68
10	1.66	1.10	24	2.87	0.03
11	1.87	(73)*	25	2.70	2.47
12	2 18	2.01	26	2.64	2.40
13	2.29	1.75	27	2.12	1.83
14	2.41	2.17	28	2.25	0.41

* The actual number of organisms is given in parenthesis. In cases where the division-rate fell below 0.00, the deficiency in animals was made up with paramecia taken from the filtered cultures, so that the seeding for the following day was again 80 animals.

Table 2 gives results with two control series, one using new filtered salt solution, the other unfiltered salt solution. Increase in the filtered medium was quite regular, but it was very inconsistent in the unfiltered medium. In the experiments cited in Table 1 the control medium was filtered each day simply as a control procedure. On the basis of results given in Table 2 and of other results, it was decided to filter all the salt medium used in subsequent experiments.

In previous work (cf. Johnson, 1933 and 1936) this difficulty with the unfiltered balanced salt solution did not exist. In this work Merck's C.P. chemicals were used in preparing the salt solution. It seems probable that there were traces of heavy metals or other substances in the salt solution, as prepared, which were adsorbed by the filter. When filtered, the salt solution was quite satisfactory for culturing *Paramecium*.

In Table 3 are given the results of two series of experiments conducted at different times under the same conditions. In Series I the controls have a slightly higher average fission-rate; in Series II the experimentals have a slightly higher average fission-rate.

Taken together, these two series of experiments do not indicate an important cumulative effect of waste products in the old medium. When double the standard suspension was used in these experiments, the results did not indicate a very marked crowding effect of bacteria, if any. Here, as before, the new cultures were seeded from the same type culture of the day before.

TABLE 3

USE OF UNFILTERED OLD MEDIUM AND 2X CONCENTRATION OF BACTERIA AT 20° C.

DAYS	SERIES I		SERIES II	
	Control	Experimental	Control	Experimental
1	1.62	....	1.32	....
2	1.12	1.00	1.71	1.06
3	0.95	1.57	0.97	1.70
4	0.88	1.08	1.87	1.74
5	1.27	1.36	1.71	1.03
6	1.53	1.66	0.61	1.39
7	2.05	1.70	0.50	1.42
8	1.91	1.66	0.23	0.29
9	0.82	1.23	1.62	2.32
10	1.59	1.68	1.09	1.14
11	2.16	1.72	1.60	1.21
12	1.88	1.72	1.18	1.13
13	1.97	1.71	0.97	0.84
14	1.98	1.77	0.92	0.99
15	2.03	1.81	1.47	0.91
16	1.45	1.11	1.09	0.84
17	1.50	1.30	0.94	0.39
18	1.50	0.35	1.56	0.87
19	1.31	1.31	0.83	0.96
20	1.51	1.28	1.27	1.22
21	1.88	1.62	1.48	1.48
22	1.77	1.55	1.00	1.86
23	1.75	1.78	0.75	1.61
24	1.89	1.81	0.76	1.33
25	1.97	1.67	1.06	1.56
26	1.94	1.72	1.36	1.43
27	1.97	1.74	1.01	1.52
28	1.80	1.92	0.89	1.45
29	1.88	1.73	0.43	1.73
30	2.12	1.87	0.64	1.72
31	1.93	1.60	1.12	1.32
32	1.88	1.37	1.68	1.69
33	1.91	1.65	1.94	1.71
34	0.81	1.35	....	....
Mean	1.66	1.52	1.13	1.27

Probability......... { Control versus experimental, Series I, $P = .0163$
Control versus experimental, Series II, $P = .1615$

Similar results were obtained in a series of cultures using 10 cc. of the standard suspension with initial seedings of 160 paramecia each day. In this case, also, the new cultures were seeded from the same type culture of the preceding day. By using this larger volume of medium, it was possible to reduce the dilution of the old medium each day. Here the amount of old medium saved each day was 9½ cc., while in 5-cc. cultures 4½ cc. were saved. This difference did not affect the nature of the results which are given as Series I in Table 4.

TABLE 4

RESULTS WITH 5- AND 10-CC. CULTURES AT 20° AND 26° C.

Days	Series I (10 Cc. at 26° C.)		Series II (5 Cc. at 20° C.)	
	Control	Experimental	Control	Experimental
1	1.57	....	1.10	....
2	1.38	1.44	1.07	0.93
3	1.96	1.75	1.23	1.84
4	2.15	2.62	1.07	1.45
5	2.35	2.72	1.03	2.34
6	2.29	2.31	1.35	0.56
7	2.30	2.49	1.75	1.61
8	2.58	2.56	2.01	2.07
9	2.51	2.63	1.95	2.19
10	1.92	2.46	2.29	2.22
11	2.42	2.47	2.21	1.84
12	2.24	2.73	2.06	1.73
13	2.33	2.56	1.86	1.56
14	3.03	2.94	1.95	1.81
15	2.67	2.60	1.99	1.88
16	2.08	2.64	1.99	1.67
17	2.59	2.61	1.26	1.07
18	2.99	2.40	1.12	0.79
19	2.77	2.92	1.60	1.42
20	2.61	2.67	1.79	1.78
21	2.80	2.93	1.76	1.73
22	2.29	2.24	1.80	1.49
23	2.70	2.84	2.39	1.71
24	2.62	2.51	1.85	1.83
25	2.48	1.75	1.77	1.22
26	2.04	2.51	1.38	1.41
27	2.14	2.12	1.35	1.19
28	2.59	2.89	1.75	1.76
29	2.80	2.51	1.83	1.43
30	2.89	2.56	1.71	1.30
31	2.27	1.34	....	....
Mean	2.42	2.46	1.69	1.53

Probability......... { Control versus experimental, Series I, $P=.1166$
Control versus experimental, Series II, $P=.1095$

In all the experiments described so far, the new cultures were seeded each day with animals from the same type of culture of the preceding day. In the experiment reported as Series II in Table 4, both experimental and control dishes each day were seeded with animals from the control dish of the day before. The failure to obtain a significant difference between the experimentals and controls in this case seems to indicate that the absence of such a difference in the preceding experiments was not due to some acclimating process in the organisms growing in the old medium.

Results comparing division-rates of cultures in X and $2X$ concentrations of bacteria at 26° C. and with all the cultures seeded from the controls of the preceding day are given in Table 5. The difference between the experimentals and controls in the $2X$ concentrations is not significant. In the case of the X concentrations, the higher fission-rate in the controls would ordinarily be taken as significant. A comparison of the two control series shows a significantly higher rate in the $2X$ concentrations. When the two experimental series are compared, a higher mean fission-rate in the $2X$ group is found, but the difference is not significant. During two of the last three days of the experiment the division-rates in the $2X$ group of experimental dishes were very low. The reason for this is not known. However, when the 2 experimentals are compared for the first 27 days, the $2X$ group has a higher rate, with a probability of .0013. Apparently, at 26° C. the $2X$ concentrations of bacteria are nearer the optimum than are the X concentrations.

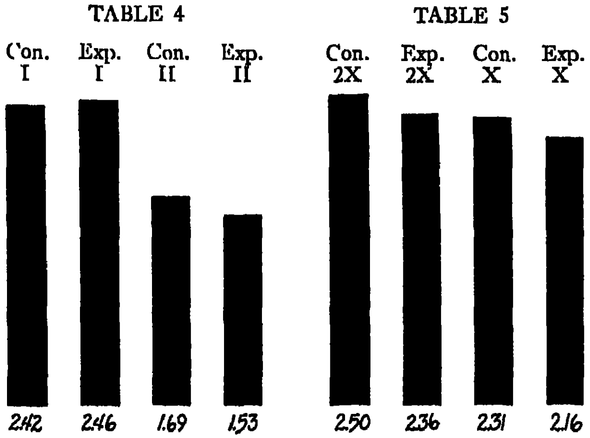


FIG. 2.—Showing the mean fission-rates given in Tables 4 and 5

Most of the earlier work, referred to above, in which the effects of waste products on reproduction were described, was carried out using hay tea or some other nutrient fluid as the culture medium. It seemed desirable to have a series comparing results obtained from hay cultures and the balanced salt cultures.

The hay medium used in these experiments was made by boiling for 3 or 4 minutes 2½ gm. of Timothy hay in a liter of buffered balanced salt solution. After cooling, the medium was passed through a porcelain filter. It was then measured out into test tubes and autoclaved. Thus, the hay medium for the whole series was uniform. In other respects the procedure was unchanged. The hay cultures, both fresh and old, were bacterized with 1 loopful of bacteria each day, just as the control cultures were.

There is no significant difference between the fission-rates of these three series. In general it can be stated that the results in the salt control series varied less than those in the two hay series.

With no definite inhibiting effect of accumulated waste products apparent in the work up to this point, there still seemed to be a possibility that the small dilution of the old medium each day, owing to the nature of the technique, might waive such effects. Two procedures to check this possibility were developed. One was to use the same medium over and over in decreasing volumes each day and thus avoid any dilution. The other was to use liquid in which large numbers of paramecia had been growing for a long time.

TABLE 5

RESULTS WITH *X* AND 2*X* CONCENTRATIONS OF BACTERIA

DAYS	2*X* CONCENTRATION		*X* CONCENTRATION	
	Control	Experimental	Control	Experimental
1	2 62	....	2.66	....
2	2.39	2.35	2.26	2.22
3	2.71	2.75	2.09	1.43
4	1 68	1.87	1.74	1.63
5	2.14	2.06	2.24	2.04
6	2.33	1.44	2.56	1.99
7	2.88	3.48	2.91	1.91
8	3.00	2.58	2.19	1.57
9	2.55	2.56	2.25	1.15
10	2 74	2.75	2 65	2.55
11	3.24	3.29	3.41	2.67
12	3.01	3.27	2.72	2.69
13	2.38	2.46	1.93	1.56
14	2.47	2.32	2.21	2.40
15	3.02	2.87	2.75	2.51
16	2.08	2.04	2.03	1.95
17	2.85	2.85	2.52	2.53
18	1.96	2.44	1.95	2.16
19	2.02	1.90	2.25	2.03
20	2.74	2.72	2.39	2.41
21	2.70	2.88	2.99	2.89
22	2.27	2.33	2.39	2.41
23	2.54	2.83	1.76	2.15
24	2.36	2.40	1.49	1.45
25	2.38	2.16	1.97	2.13
26	2.89	2.74	2.85	2.67
27	2.32	1.52	1.73	2.38
28	2.44	2.48	2.62	2.40
29	2.86	1.10	2.49	2.69
30	1.71	0.15	1.89	2.02
31	2.43	2.24	2.24	2.10
Mean	2.50	2.36	2.31	2.16

Probability.......
2*X* control versus 2*X* experimental, $P = .1095$
X control versus *X* experimental, $P = .0278$
2*X* control versus *X* control, $P = .0026$
2*X* experimental versus *X* experimental, $P = .1615$

The control culture reported in Table 7 was started with 10 cc. of the standard suspension and seeded with 160 paramecia. The liquid (9½ cc.) from this culture was used as the experimental medium on the second day and was seeded with 152 paramecia. The control culture of the second day had the same amount of fluid and the same number of organisms. On each succeeding day the volume of the cultures was reduced ½ cc., and the seeding of organisms was 8 less than the preceding day. The volume of the cultures on the last day was 2 cc. With the volumes of the cultures and the seedings of animals proportional throughout, the fission-rates were quite constant. There was no significant difference between the experimentals and the controls for the experiment as a whole.

TABLE 6

COMPARING BACTERIZED HAY MEDIUM WITH SALT CONTROL AT 26° C.

Days	Salt Control	Fresh Hay	Old Hay	Days	Salt Control	Fresh Hay	Old Hay
1...........	2.19	2.74	....	17..........	1.84	1.42	1.97
2...........	2.24	2.29	1.46	18..........	1.91	1.14	2.05
3...........	2.13	2.93	0.66	19..........	2.90	1.03	2.54
4...........	1.97	2.49	1.28	20..........	2.77	1.95	2.18
5...........	1.79	2.87	1.72	21..........	2.15	2.39	1.73
6...........	2.16	2.35	2.21	22..........	2.17	3.09	2.66
7...........	2.76	3.02	2.85	23..........	1.88	2.09	2.53
8...........	1.99	2.82	2 56	24..........	2.51	0.88	3.13
9...........	1.81	1.56	2 38	25..........	2.67	3.00	2.21
10...........	2.51	3.04	2.66	26..........	2.38	3.23	2.41
11...........	2.34	2.88	2 46	27..........	2.65	3.22	3.01
12...........	1.98	2.41	2.03	28..........	2.38	1 44	2.57
13...........	2.33	2.62	1.86	29..........	2.74	2.03	3.46
14...........	1.53	2.77	3.34	30..........	2.32	2.71	2.57
15...........	1.84	1.49	2.20				
16...........	1.62	2.25	2.18	Mean...	2.21	2.32	2.30

Probability............. { Fresh hay versus old hay, $P = .5203$
Old hay versus salt control, $P = .4237$
Fresh hay versus salt control, $P = .2301$

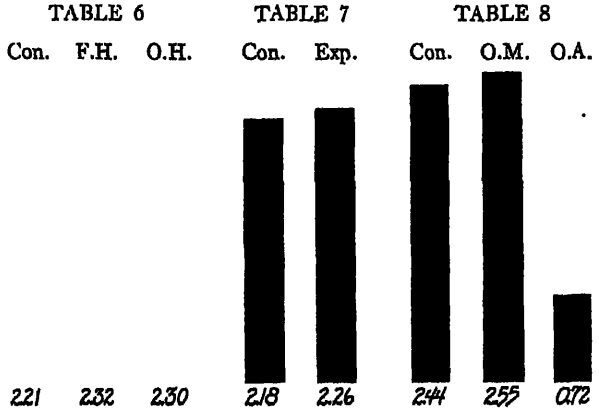


FIG. 3.—Showing the mean fission-rates given in Tables 6, 7, and 8

In order to have an old medium in which paramecia had been growing and living in large numbers for a long time for use in these experiments, two 1-liter flasks of buffered salt solution were seeded, each with a single *Paramecium*. One of these liter cultures has been inoculated with 10 heaping loopfuls of *P. fluorescens* every day since its beginning. The other one has been inoculated with the same amount of bacteria every other day. In each case the population gradually increased until a stationary level in numbers was reached. In the first case a level of approximately 225 organisms per cubic centimeter was reached after 20 days, and has been maintained for over 4 months. In the second case a level of approximotely 175 was reached after 29 days, and here also the numbers have remained almost constant. A detailed study of these populations will be the subject of another paper.

Five cubic centimeters of medium from one of the liter cultures which had been freed of all paramecia were used as the old medium in each of the four series given in the upper section of Table 8. A standard loopful of bacteria was used in each culture. The seedings of both the control cultures and the old-medium cultures were made from the control culture of the preceding day. The old medium varied in age from 29 to 108 days; yet there was no significant difference between the controls and the old-medium cultures. Quite a different situation existed in the fresh salt-solution cultures seeded with paramecia taken from the liter flasks. In all four cases the fission rates were less than 1.00. The results obtained using control animals in the old medium indicated that there was nothing in the old fluid which inhibited growth and division. The difference here seems to be due to a difference in physiological state between the control animals and those in the liter cultures. Actually, the paramecia in the liter cultures were in a state of semistarvation. By taking a 5-cc. sample from each of the liter flasks after the daily count and feeding, and checking the increase at the end of 18 hours, an indication of the normal rate of increase in the flasks was obtained. In the case of the sample taken from the liter flask which was fed daily, the increase after 18 hours was 0.22. In the other sample from the flask which was fed every other day, the rate of increase after 18 hours was 0.32. The 5-cc. control cultures were fed 1 loopful of bacteria daily. The 1,000-cc. cultures were fed 10 loopfuls of bacteria either daily or every other day. The results given in the lower section of Table 8 show that the division-rates of the paramecia taken from the liter cultures had not been permanently lowered. In one case, Series II, the

TABLE 7

USE OF UNDILUTED OLD MEDIUM AT 26° C.

Days	Control	Experimental	Days	Control	Experimental
1	2 14	....	10	2.37	2.58
2	1 73	1.92	11	2.34	2.11
3	2.27	2.57	12	1.89	1.15
4	2.21	2.26	13	2.12	2.13
5	2.31	2.42	14	1.93	2.44
6	2.25	2 49	15	2.49	2.35
7	2.29	2.82	16	2.18	2.06
8	2.13	2 32	17	2.25	2.22
9	2.19	2.32			
			Mean	2.18	2.26

Probability: Experimental versus control, *P* = .2886 cultures were continued for 5 days with the medium changed daily. On both the fourth and fifth days the liter-animal cultures were about equal to the controls. In another case, Series III, after the first day the liter animals were placed in 5 cc. of medium taken from the liter flasks, and this was used over again on the succeeding days. Here, also, the fission rates of the experimentals came up to that of the controls. These results seem to indicate that a period of acclimation is necessary after *Paramecium* has been in a state of semistarvation for a long period of time.

TABLE 8

USE OF OLD MEDIUM FROM 1-LITER CULTURES AT 26° C.

Series	Age in Days	Control	Control Animals in Old Medium	Animals from Old Cultures in Fresh Salt
I	29	2.52	2.52	0.55
II	43	2.54	2.49	0.59
III	49	2.48	2.35	0.89
IV	108	2.24	2.86	0.88
Mean		2.44	2.55	0.72

Probability......
- Control versus control animals in old medium, $P = .5740$
- Control versus animals from old culture, $P = .0000$

Days	From Series II (Medium Changed Daily)		From Series III (Old Medium Used Over)	
	Control	Experimental	Control	Experimental
1	2.54	0.59	2.48	0.89
2	2.64	0.52	2.50	1.12
3	2.05	1.79	2.41	2.10
4	2.90	2.61	2.01	2.16
5	2.58	2.57	2.09	2.13

To obtain bacteria-conditioned medium, a normal loopful of bacteria was added to a dish containing 5 cc. of the salt solution for a number of days. This medium was then used for culturing *Paramecium*. Table 9 shows the results obtained using such conditioned medium of different ages. All the bacteria-conditioned medium used inhibited growth of the paramecia, the older the medium the greater the inhibition. In most of the 30-day cultures some of the paramecia died. Both the filtering through a porcelain filter and the autoclaving of this medium improved it some, but even then it was quite inferior to the normal salt solution. Filtering showed that this depressing effect was not entirely due to bacterial crowding of the paramecia.

Bacterial counts on control cultures at the end of the 24-hour period showed that the original density had been reduced to from 2 to 16 per cent of the original density in different counts made. Thus, most of the bacteria were being used up each day. Under such conditions, and with some of the remaining bacteria removed in the centrifuging, the old culture medium which was used over and over would not accumulate excess bacteria rapidly. It seems probable, both from the appearances of the cultures and from the amount of bacteria inoculated for the number of organisms, that the old medium used from the liter cultures was relatively free from bacteria at the time it was removed.

TABLE 9

USE OF BACTERIA-CONDITIONED MEDIUM

Length of Conditioning (Days)	Conditioned	Control
15	1.47	2.69
20	0.77	2.32
20	0.14	2.59
30	(17)*	2.47
30	(78)*	2.24
30	(61)*	2.56
30	(64)*	2.01
30 (hay)	0.14	2.32
30 (filtered)	1.21	2.35
30 (autoclaved)	0.99	2.20
30 (1 cc.+4 cc. of salt solution)	1.42	2.09

* The actual numbers of *paramecia* are given in parentheses.

TABLE 10

SUMMARIZING TABLE

TABLE NO.	MEAN DIVISION-RATES		TABLE NO.	MEAN DIVISION-RATES	
	Control	Experimental		Control	Experimental
I	1.81	1.66	V (2X)	2.50	2.36
III (1)	1.66	1.52	V (X)	2.31	2.16
III (2)	1.13	1.27	VI	2.21	2.30
IV (1)	2.42	2.46	VII	2.18	2.26
IV (2)	1.69	1.53			

Probability: Control versus experimental, $P = .3944$

DISCUSSION

The results described above are not in agreement with the results obtained by most of the earlier workers on protozoan populations concerning the effects of waste products on reproduction. They are, however, essentially like the recent results of Phelps (1936) and of Taylor and Strickland (1938). In only two of the nine paired series reported were the control fission-rates significantly higher than the experimental ones. A comparison of the mean division-rates of the experiments as a whole is given in Table 10. The

P-value given here indicates no significant difference in division-rate between the cultures grown in fresh medium and those grown in old medium for the experiments as a whole.

The investigators who reported an inhibiting effect of waste products on reproduction in from 24 to 48 hours used hay tea or some other nutrient medium with unknown mixed bacteria as the source of food. In these experiments when hay medium was used over and over again for cultures but with a fresh supply of a known kind of bacterium added each day, reproduction comparable to the daily fresh controls was obtained. In view of these findings it seems probable that workers who described effects of waste products in from 24 to 48 hours were dealing with a situation in which the protozoans had utilized most of the bacteria suitable as food for them in that period of time and thus exhibited a declining fission-rate because of the lack of food. The fact that at the end of a 48-hour period numerous bacteria are present in a protozoan culture, when the kinds of bacteria present are unknown, is no proof that those bacteria are suitable as food for the particular protozoan being cultured.

The ideas suggested by Phelps (1936) should not be ignored. It has been shown here that medium conditioned with a single kind of bacteria for a number of days does inhibit reproduction of *Paramecium*. His suggestion, that the inhibiting effects described by several investigators might have been due to the accumulated wastes of the mixed bacteria in the cultures, should be tested with actual experiments. It would appear, from this work, that such effects would not be noticeable in such short periods of time. His second suggestion, that with different sources of food the same protozoan might produce entirely different waste products, also needs testing.

The results of Dimitrowa (1932) might be explained by the hypothesis that she changed the bacterial flora significantly when she added small amounts of old culture liquid to new medium.

The conclusion reached here, that, with the culture conditions used, the accumulated waste products in old culture medium did not significantly lower the reproductive rate of *Paramecium*, does not preclude entirely such effects under other conditions or for criteria other than reproduction-rate. Such questions as "Are the waste products of such cultures, in the main, volatile substances which readily escape from the medium?" "Are these waste substances very unstable materials which are changed by the associated organisms in the culture and thus rendered unharmful?" and "Are the waste products adsorbed by the dead organisms or secretions of the organisms in the culture and thus made ineffective?" are suggested; but they cannot be answered here.

The changes in the H-ion concentration of all of the cultures studied were slight and consistent. The medium tested after 24 hours, old medium tested after 30 days of use, old medium taken from the liter cultures, and 30-day-old bacteria-conditioned medium tested pH 7.2 in all cases.

As was to be expected, cultures grown at 26° C. had a consistently higher fission-rate than those grown at 20° C.

SUMMARY

1. For the conditions described, there was no significant inhibiting effect on the reproductive rates of *P. multimicronucleata* grown in old culture medium with the waste products supposedly accumulating.

2. Similar results were obtained both in the use of bacterized balanced salt medium and in bacterized hay medium.

3. The seeding of the old-medium cultures with organisms from the preceding day's old-medium culture over and over gave results which were very comparable to those obtained in old-medium cultures which were seeded each day from control cultures started the preceding day.

4. Paramecia which have lived in a condition of semistarvation for a period of time and which have been reproducing with a very low division-rate will, when placed in medium containing adequate food, resume a normal rate of division after a period of 2 or 3 days.

5. Medium conditioned with the bacterium *P. fluorescens* for 15–30 days when used for the culture of *Paramecium* definitely inhibits reproduction.

6. Changes in H-ion concentration in all of the cultures were slight and consistent.

7. Higher fission-rates were obtained at 26° C. than at 20° C., and $2X$ concentration of bacteria was nearer the optimum at 26° C. than X concentration.

LITERATURE CITED

ALLEE, W. C. 1931. Animal aggregations. Chicago: University of Chicago Press Pp. 431.

BEERS, C D. 1933. The relation of density of population to rate of reproduction in the ciliates *Didinium nasutum* and *Stylonychia pustulata*. Arch f Protistenk., 80:36–64.

CALKINS, G. N. 1926. Biology of the protozoa. Philadelphia: Lea & Febiger. Pp. 625.

CURRAN, H. R. 1925. The growth of bacteria Thesis, Cornell University.

DIMITROWA, A. 1932. Die fordernde Wirkung der Exkrete von *Paramoecium caudatum* Ehrbg. auf dessen Teilungsgeschwindigkeit. Zool. Anz., 100:127–32.

DI TOMO, M. 1932 Richerche sul comportamento di *Paramecium caudatum* in un dato volume di cultura liquida. Boll. di zool., 3:137–40

EIJKMAN, C. 1907. Über die Ursache der Wachstumhemmung in Bacterienkulturen. Deutsche med. Woch., 33:265–66.

GAUSE, G. F. 1934. The struggle for existence. Baltimore: Williams & Wilkins Co. Pp. 163.

GREENLEAF, W. E. 1926. The influence of volume of culture medium and cell proximity on the rate of reproduction of Infusoria. Jour. Exper. Zool., 46:143–69.

HENRICI, A. T. 1928. Morphologic variation and the rate of growth of bacteria. Springfield, Ill.: C. C. Thomas Pp. 194.

JAHN, T. L. 1934. Problems of population growth in the protozoa. Collecting Net, 9:34–41, 47–48.

JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zool., 6:22–54.

———. 1936. Studies on the nutrition and reproduction of *Paramecium*. *Ibid.*, 9:1–14.

———. 1937. Experimental populations of microscopic organisms. Amer. Nat., 71:5–20.

MYERS, E. C. 1927. Relation of density of population and certain other factors to survival and reproduction in different biotypes of *Paramecium caudatum*. Jour. Exper. Zool., 49:1–43.

PETERSEN, W. 1929. The relation of density of population to rate of reproduction in *Paramecium caudatum*. Physiol. Zool., 2:221–54.

PHELPS, AUSTIN. 1936. Growth of protozoa in pure culture. II. Effect upon the growth curve of different concentrations of nutrient materials. Jour. Exper. Zoöl., 72:479–96.

"STUDENT," 1925. New tables for testing the significance of observations. Metron, 5:105–20.

TAYLOR, C. V., and STRICKLAND, A. G. R. 1938. Reactions of *Colpoda duodenaria* to environmental factors. I. Some factors influencing growth and encystment. Archif. f. Protistenk. (in press).

WOODRUFF, L. L. 1911. The effect of excretion products of *Paramoecium* on its rate of reproduction. Jour. Exper. Zoöl., 10:551–81.

STUDIES ON THE PHYSIOLOGY OF AMOEBA. II. THE ALLELOCATALYTIC EFFECT IN AMOEBA CULTURES FREE OF BACTERIA

(Four figures)

KARL REICH
Department of Zoölogy, Hebrew University, Jerusalem

SINCE the phenomenon of allelocatalysis was first described by Robertson (1921), it has been intensely discussed in the protozoölogical literature; however, no definite conclusion has been reached in this discussion. As the earlier results were dealt with in detail by Allee (1931), Beers (1933), and Johnson (1933), I may confine myself to giving a brief summary, which is necessary for clearing up the present state of the problem. The earlier publications on allelocatalysis may be classed under three headings. The first confirms the phenomenon of allelocatalysis. Besides Robertson's work on *Enchelys farcimen* (1921), *Colpoda* sp. (1924 *a*, *b*), and the long series of his publications dating up to 1927, this group comprises only Yocom's paper on *Oxytricha* (1927) and Petersen's on *Paramaecium caudatum* (1929). Petersen's results deserve our special attention, as she demonstrated the importance of the relation between the volume of the animals and that of the medium. Her results, while negative for cultures in 1 drop of nutrient solution, were positive in media of 20–40 drops.

The second group, including the work with negative result, is far larger. Cutler and Crump (1923) showed that in *Colpidium colpoda* the division rate of cultures with a larger initial number of individuals does not exceed that of cultures with smaller initial numbers. In 1925 (in answer to the objections of Robertson [1924]), they confirmed their result for well-washed infusoria. Greenleaf (1926) investigated *P. aurelia*, *P. caudatum*, *Pleurotricha lanceolatum*, and *Stylonychia pustulata* and arrived at negative conclusions in all cases. Similarly, negative results were obtained by Calkins (1926, p. 206), for *Uroleptus mobilis*, by Myers (1927) for *P. caudatum*, Grimwald (1928) for *C. colpoda*, and Jahn (1929) for *Euglena*. Among the more recent investigators, Beers (1933) also arrived at negative results for *Didynium nasutum* and *S. pustulata*. In order to prevent any possible modification of the result by bacteria, he employed inorganic solutions as media, and in one case *Paramaecium*, in another *Chlamydomonas*, as food. However, he did not take into account that the allelocatalytic effect is not species-specific (according to Petersen, 1929) and that, consequently, the presence of a relatively large number of food animals in these cultures was bound to preclude the detection of allelocatalysis. Lastly, Phelps (1935), investigating mass cultures of *Glaucoma pyriformis*, free of bacteria, concluded that "experiments on *Glaucoma* do not support the theory of allelocatalysis."

This summary of negative results does not deal with the frequently quoted experiments of Peskett (1924, 1925), as these were carried out on yeast, an object possessed of many biological peculiarities rendering the interpretation of the results extremely difficult.

The third group includes publications that confirm the increased division rate in cultures with larger inoculum but try to account for this phenomenon by changes in the medium. The first to be mentioned here are the papers adducing the bacteria as the factor determining the observed variations, i.e., those of Cutler and Crump (1924) on *C. colpoda*, that of Chejfec (1929) on *P. caudatum* and of Johnson (1933) on *Oxytricha fallax* and 1936 on *P. caudatum*. They showed to what extent the growth of the cultures is influenced by the bacteria, and concluded that the phenomenon termed "allelocatalysis" is an effect of the variations in the number of bacteria. On the other hand, a theory put forward by Darby (1930) assumes that the ciliates modify the pH of their nutrient medium in optimum direction, thus producing a more favorable reaction in the presence of a higher number of animals per volume.

Briefly summarizing the literature, we must admit that in most cases an allelocatalytic effect was not proved at all, and that such few results as were positive could be explained by accessory circumstances involved by the conduction of the experiment, without necessitating the assumption of an allelocatalytic theory.

When I decided, despite these facts, to undertake the experiments to be described, I was determined by the following reasons. Anyone working on protozoan cultures may easily experience that the success of the culture is, to a high degree, dependent on the size of the inoculum. Cultures with a large amount of initial material will succeed more readily than those with a smaller amount. The latter will frequently grow scantily in the beginning and, after a more or less prolonged interval, start accelerated growth, with a jerk. Similar phenomena may be observed in cultures of algae or bacteria. Lastly, the conditions in tissue cultures, as described by Fischer (1933), should be mentioned.

Moreover, as the material at my disposal allowed of mass culturing, a technique that has been employed in only few allelocatalytic experiments up to the present, I felt that it was worth while, despite the numerous negative results, to investigate the problem once more.

MATERIAL AND METHODS

All experiments were performed on cultures of *Mayorella palestinensis*, a soil amoeba, the cultivation of which is described in a previous paper (Reich, 1935). Great care was exercised to eliminate all disturbing accessories. The series of cultures to be compared were all inoculated into the same nutrient solution, using the same mother culture. The test tubes used in corresponding experiments were all of the same glass (in one part of the experiments, Jena glass; in another part, Pyrex). The diameters were as nearly equal as possible, so as to avoid the disturbing influence of different heights of liquid columns. The tubes were filled with 4.5 cc. of nutrient solution and sterilized in the autoclave. Well-growing young amoeba cultures were centrifuged, washed four times with a 0.1 per cent solution of NaCl, and diluted with the same solution to the requisite concentrations of amoebae. Of the suspension thus prepared, 0.5 cc. per culture were inoculated. Before taking out a sample, part of the liquid was sucked up into the pipette and then powerfully evacuated. This process was repeated several times so as to insure that the amoebae were distributed as uniformly as possible. The eddies thus produced were sufficient to keep afloat such small-sized animals as the amoebae. In view of the smallness of the animals and of the necessity to work under sterile conditions, direct counting of the amoebae had to be dispensed with. Table 1 gives a survey of the result of counting an amoeba suspension and the five cultures inoculated with it. Counting was performed in the Thoma blood-counting chamber. After thoroughly shaking the cultures, 10 drops of each were counted. As five cultures were made for every grade of dilution, the figures in the tables represent eleven average values of 50 counts and refer to 0.1 cu. mm. of nutrient solution.

TABLE 1

THE RESULT OF COUNTS IN NUMBER OF ANIMALS IN A SUSPENSION OF AMOEBAE AND IN THE CULTURES MADE UP FROM IT

	NUMBER OF AMOEBAE IN EACH OF TEN COUNTS										AVERAGE OF TEST TUBE	AVERAGE OF THE FIVE CULTURES
	1	2	3	4	5	6	7	8	9	10		
Suspension	9	9	6	9	8	8	13	4	9	5	8.0	
Culture 1	1	1	1	3	1	0	0	0	2	1	1.0	0.78
2	0	0	2	0	2	1	0	0	2	0	0.7	
3	2	0	1	0	1	1	0	0	1	1	0.7	
4	1	0	2	0	1	1	1	1	1	0	0.8	
5	1	1	0	0	2	0	0	1	0	2	0.7	

In the experiments with extracts, only 4 cc. of nutrient solution were used and 0.5 cc. of the extract or, in the controls, 0.5 cc. of the extracting liquid were added. The pH of the cultures was registered before and after inoculation and was found to be unaffected. All cultures were kept in a thermostat at 28° C. In view of the slow growth of the cultures it was found sufficient to count them after every 48 hours. Where cysts occurred, counting was given up, as such cultures will not yield any comparable values.

DEVELOPMENT OF THE CULTURES

In order to survey the development of any normal culture, we choose a random example, as represented in Table 2 and Figure 1. Considering first of all the increase in amoeba number, we find that within the first 48 hours after inoculation there is but a slow rise. This is followed by a short period of relatively rapid growth, which gradually passes over into a phase of ever decreasing rate of multiplication. This outline corresponds to the scheme of growth described by Phelps (1935), except that the final stationary phase is not represented in my example. It was omitted since it sets in simul-

TABLE 2

THE DEVELOPMENT OF A NORMAL CULTURE

	NUMBER OF DAYS OF CULTURING										
	0	2	4	6	8	10	12	14	16	18	20
Number of amoebae	1.0	1.4	3.9	7.3	9.0	14	19	21	26	26	29
Rate of division per 48 hours		0.5	1.5	0.9	0.3	0.7	0.4	0.1	0.3	0.0	0.2
Total number of steps of division		0.5	2.0	2.9	3.2	3.9	4.3	4.4	4.7	4.7	4.9

taneously with the formation of cysts. During the prolonged subsequent period the number of amoebae fluctuates faintly about a constant value, whereas the number of cysts rises continuously. It is, of course, hard to decide whether the cysts are to be entirely neglected in the definite figuring of the population maximum or to be taken into account, and if so, in what form. This question, being unessential for the problem of allelocatalysis itself, has been left open, and the counts were stopped at the appearance of cysts.

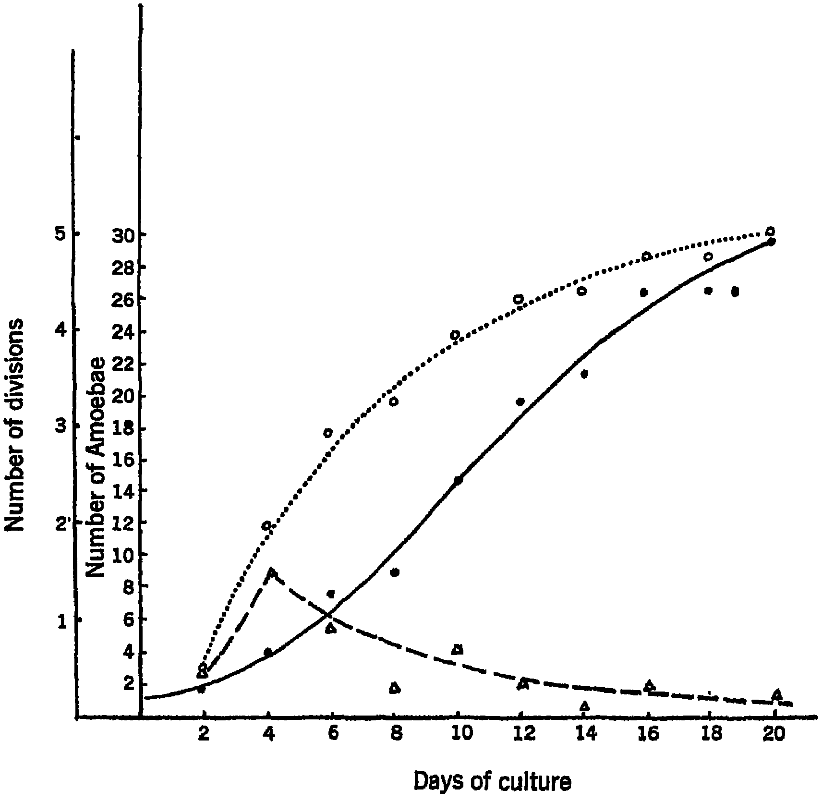


FIG. 1.—Development of an amoeba culture. The number of amoebae, ●——●; the rate of division in each period of 48 hours, △----△; the sum of steps of division since the beginning of the culture, O······O.

The development of the same culture may be represented in another way. If the rate of division for each particular period of 48 hours is worked out, it is seen that to the slow initial increase in cell number there corresponds a low rate of division, which subsequently rises steeply and then, slowly and gradually, declines. If the total number of steps of division since the beginning of the culture after every 48 hours is worked out and plotted, the curve obtained rises steeply in the beginning and then more and more approaches the horizontal.

After considering which of these modes of representation would be best suited to reveal the presence or absence of allelocatalysis, we selected the last mentioned method.

This offers the advantages, first, of automatically eliminating accidental fluctuations of the individual counts, and, second, of yielding relative values, which are, in the beginning of the curve, independent of the absolute number of amoebae present in the culture. In the absence of allelocatalysis the initial parts of the curves of one experiment will be identical, unaffected by the absolute number of individuals inoculated in the different series of cultures. On the other hand, if allelocatalysis is at work, this part of the curve will be the less steep, the smaller the inoculum employed. The end of the curve is dependent on the number of amoebae in both cases and will be the more nearly level the more nearly a maximum population density will be approached.

EXPERIMENTS ON ALLELOCATALYSIS

The first experiment was conducted in three series (*a*, *b*, *c*,) each of which comprised five test tubes. The results are shown in Table 3 and Figure 2. The series *a* started off with a concentration of 1 amoeba per 0.1 cu. mm. of nutrient solution. In the first 48 hours the division rate amounted to 0.5; later it rose to a maximum of 1.5, and then decreased slowly and gradually. The maximum population density attained on the eighteenth day of culturing came to 26 amoebae per 0.1 cu. mm. of solution. Series *b* began with 0.7 amoebae per 0.1 cu. mm. of solution. The rate of division lagged at first somewhat behind that of series *a*, but then rose quickly so as to reach the total number of steps of series *a* on the tenth day. The experiment was interrupted on the eighteenth day of culturing, when the numbers of amoebae in the two series were equal. But as the initial number of series *a* exceeded that of series *b*, the total number of steps of division in the latter was higher (5.2) than in the former (4.7). The third series started with a concentration of 0.3 amoebae per 0.1 cu. mm. The rate of division was very low in the beginning, and more vigorous multiplication did not set in before the tenth day. The experiment was broken off before this series had attained its maximum density.

TABLE 3

THE DEVELOPMENT OF THE THREE SERIES (*a*, *b*, AND *c*) OF EXPERIMENT NO. 1

DAYS OF CULTURING	NUMBER OF AMOEBAE			RATE OF DIVISION PER 48 HOURS			TOTAL OF STEPS OF DIVISION		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
0	1.0	0.7	0.3	....	....	....	....	....	....
2	1.4	1.0	0.4	0.5	0.5	0.4	0.5	0.5	0.4
4	3.9	1.7	0.3	1.5	0.8	....	2.0	1.3	0.4
6	7.3	4.3	0.5	0.9	1.2	0.4	2.9	2.6	0.8
8	9.0	5.3	0.8	0.3	0.3	0.6	3.2	2.9	1.4
10	14	10	1.7	0.7	1.0	1.0	3.9	3.9	2.4
12	19	14	2.9	0.4	0.5	0.8	4.3	4.4	3.2
14	21	18	5.6	0.1	0.4	0.9	4.4	4.7	4.2
16	26	20	8.2	0.3	0.1	0.5	4.7	4.8	4.7
18	26	26	11	0.0	0.4	0.5	4.7	5.2	5.2

In the second experiment (Table 4 and Fig. 3) the initial concentrations were 0.6, 0.2, and 0.04 amoebae per 0.1 cu. mm. in series *a*, *b*, and *c*, respectively. The development of the cultures resembled that of experiment No. 1. The rate of division in series *a* was highest at the very beginning and afterward fell off gradually; in series *b* the curve was distinctly less steep in the beginning, and the rate of division reached its maximum

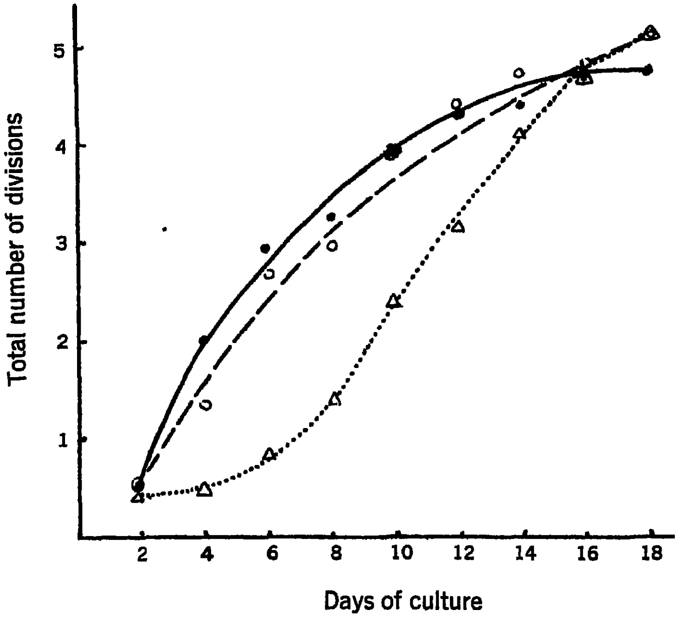


FIG. 2.—The sum of steps of division in the three series of experiment No. 1. Initial populations, given as number of amoebae per 0.1 cu. mm. of medium, as follows: series *a*, ●——●, 1.0; series *b*, ○– – –○, 0.7; series *c*, △······△, 0.3.

TABLE 4

THE DEVELOPMENT OF THE THREE SERIES (*a*, *b*, AND *c*) OF EXPERIMENT NO. 2

DAYS OF CULTURING	NUMBER OF AMOEBAE			RATE OF DIVISION PER 48 HOURS			TOTAL OF STEPS OF DIVISION		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
0	0.6	0.2	0.04	....	....	....	....	....	....
2	1.9	0.4	....	1.5	0.8	....	1.5	0.8	....
4	4.5	0.6	0.08	1.2	0.7	1.0	2.8	1.5	1.0
6	7.8	1.0	0.08	0.8	0.7	0.0	3.6	2.2	1.0
8	12	1.0	0.04	0.6	....	....	4.2	2.2	1.0
10	14	2.5	0.16	0.3	1.4	1.0	4.5	3.6	2.0
12	21	5.3	0.08	0.5	1.1	....	5.0	4.7	2.0
14	....	10	0.28	....	0.9	0.8	....	5.6	2.8
16	....	14	0.24	....	0.5	....	....	6.1	2.8
18	....	18	0.28	....	0.4	0.0	....	6.5	2.8
20	....	....	0.28	....	....	0.0	....	....	2.8
22	....	....	0.64	....	....	1.2	....	....	4.0
24	....	....	1.8	....	....	1.5	....	....	5.5
26	....	....	3.2	....	....	0.8	....	....	6.3
28	....	....	7.1	....	....	1.2	....	....	7.5

not before the tenth day; while in series *c* the curve was almost level in the beginning, and a more intensive and regular multiplication set in only about the twentieth day.

Experiment No. 3 (Table 5 and Fig. 4) was performed in two series with initial concentrations of 0.4 amoebae in series *a* and 0.04 in series *b*. The results again show, in series *a* maximum multiplication in the first days and slow decrease during the subsequent period, whereas in series *b* intense multiplication starts only toward the end of the time of the experiment.

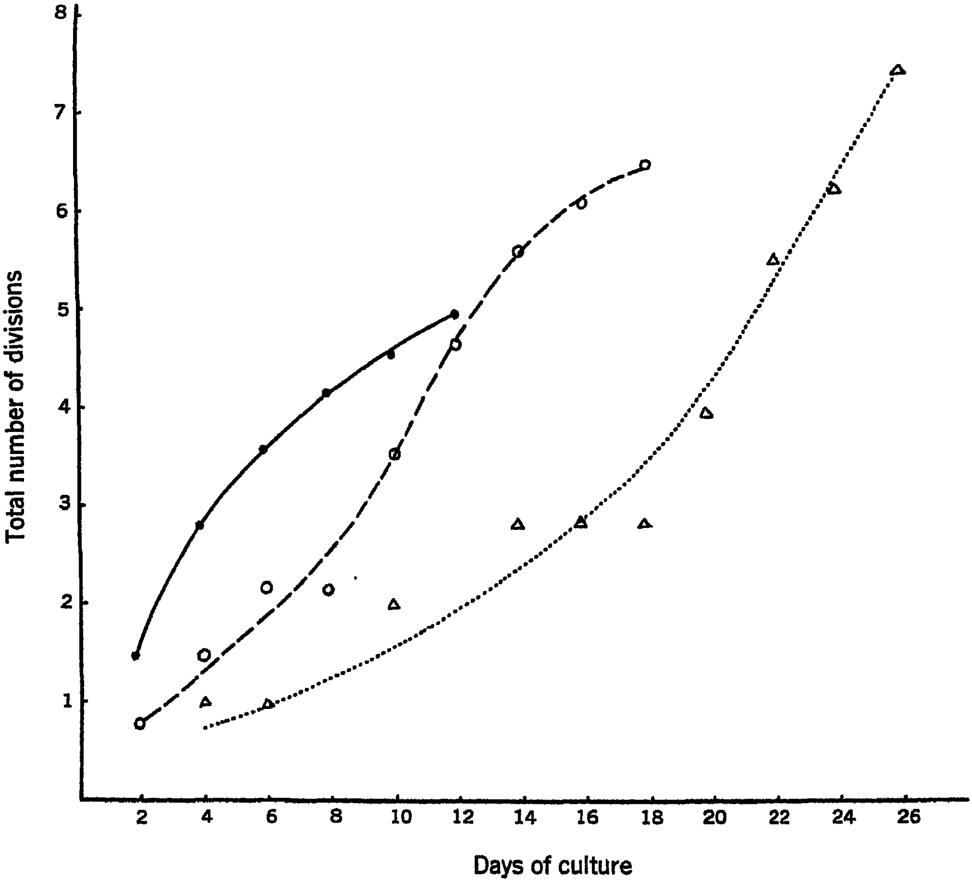


Fig. 3.—The sum of steps of division in the three series of experiment No. 2. Initial populations, given as number of amoebae per 0.1 cu. mm. of medium, as follows: series *a*, ●——●, 0.6; series *b*, O——O, 0.2; series *c*, △·····△, 0.04.

A fourth experiment with the same concentrations offered a similar aspect, although the rate of division rose somewhat earlier in series *b*, so as to attain its maximum already between the sixteenth and eighteenth days.

Surveying the total result of all the four experiments, we must state the fact that in all cases the series with lower initial concentrations of amoebae showed a much smaller initial rate of division than the cultures with higher initial concentrations under identical

TABLE 5

THE DEVELOPMENT OF THE TWO SERIES (*a* AND *b*) OF EXPERIMENT NO. 3

Days of Culturing	Number of Amoebae		Rate of Division per 48 Hours		Total of Steps of Division	
	a	*b*	*a*	*b*	*a*	*b*
0	0.4	0.04	......	......	......	......
2	1.7	0.00	2.1	......	2.1	......
4	4.6	0.16	1.4	2.0	3.5	2.0
6	8.1	0.16	0.8	0.0	4.3	2.0
8	14	0.16	0.9	0.0	5.2	2.0
10	16	0.24	0.1	0.6	5.3	2.6
12	......	0.4	......	0.7	......	3.3
14	......	0.56	......	0.5	......	3.8
16	......	0.96	......	0.7	......	4.5
18	......	1.2	......	0.3	......	4.9
20	......	1.7	......	0.5	......	5.4
22	......	2.0	......	0.2	......	5.6
24	......	3.3	......	0.7	......	6.3
26	......	9.0	......	1.5	......	7.8

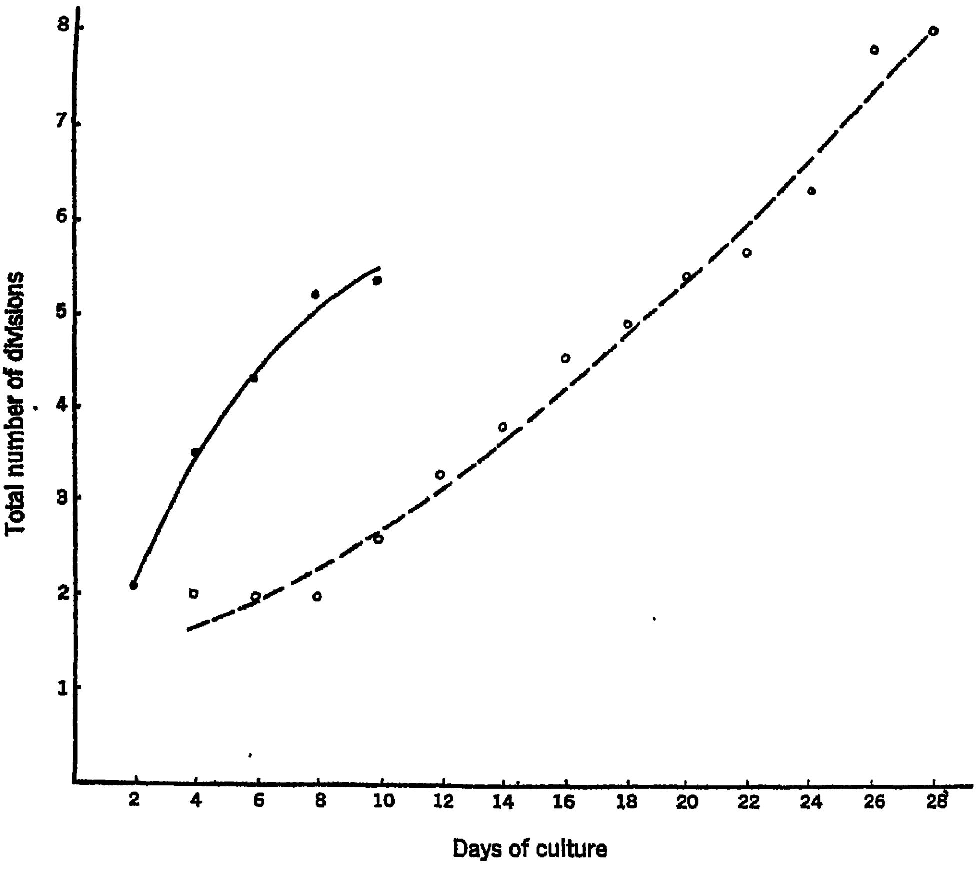


FIG. 4.—The sum of steps of division in the two series of experiment No. 3. Initial populations, given as number of amoebae per 0.1 cu. mm. of medium, as follows: series *a*, ●——●, 0.4; series *b*, ○----○, 0.04.

conditions. While in the highest concentrations the maximum rate of division was attained immediately or soon after inoculation, decreasing slowly with rising concentrations, the series with scanty initial populations showed a small rate of division in the beginning, which increased as the population grew denser, and fell off after a certain maximum density was reached. This phenomenon became fully manifest in those cultures that started off with a population below 0.3 amoebae per 0.1 cu. mm. medium. This behavior confirms the fundamental assumption of the allelocatalytic theory, i.e., that living cells exercise a mutual influence on their multiplicative activity, causing it to rise with increasing population density up to a certain point, after which it remains independent of population number for a time, until it falls off when the optimum density has been exceeded.

EXPERIMENTS WITH VARIOUS EXTRACTS

Robertson's explanation for the allelocatalytic phenomenon refers it to the action of certain substances diffusing from the nuclei into the surrounding medium. If this is correct, it must be possible to increase the division rate by using nutrient solutions in which amoebae have previously multiplied. This assumption was to be checked by a large series of experiments.

The first experiment was carried out with solutions in which amoebae had been kept for various lengths of time. The solutions were prepared by inoculating test tubes, counting daily, and centrifuging when the requisite density of amoebae was reached. The nutrient solution in the control tubes had undergone similar treatment, except that, in place of inoculating by amoeba suspension, it was diluted with NaCl solution. Measured quantities of the supernatant nutrient solution were pipetted off and distributed into sterile test tubes. All tubes were then inoculated with the same culture. This experiment included seven series, in which the nutrient solutions had been centrifuged off at various amoeba densities from 0.9 to 13 individuals per 0.1 cu. mm. solution. The cultures in whose medium amoebae had previously grown did not differ in any appreciable manner from the controls.

In the next experiment four series of tubes were inoculated with different amoeba concentrations so as to produce initial densities of 1.3, 0.4, 0.08, and 0.04 amoebae per 0.1 cu. mm. (0.0 amoebae in the controls). After 24 hours all cultures were centrifuged, the supernatant solutions of each series were separately collected, and measured volumes were transferred to sterile test tubes. Subsequently, the tubes of all series were inoculated with one amoeba culture. As in the previous instance, the new cultures did not exhibit any effect of the former existence of amoebae in the medium.

In view of all those negative results it remained to be seen if the hypothetically active substance might be extracted from the animals themselves. Ten young cultures were centrifuged, and the sediment was then left for 3 hours in 10 cc. of NaCl solution. This extract was added to the nutrient solution and did not prove more effective than the control NaCl solution.

Since with plant auxines chloroform extraction yields particularly good results, this method was also tested. The liquid of fifty cultures was centrifuged off, and 5 cc. of chloroform were added to the sediment. After 48 hours the chloroform was pipetted off and passed through sterile filter paper. By allowing the filtrate to evaporate at room temperature, a solid residue was obtained, which was taken up in 5 cc. of nutrient solution. Of the extract thus prepared, 0.5 cc. was added to every 4 cc. of nutrient solution.

There were two series of controls. (1) The centrifuged liquid medium of twenty-five cultures was shaken up with 5 cc. of chloroform, and the chloroform pipetted off after 48 hours and further treated as above described. (2) Five cubic centimeters of chloroform were filtered and evaporated, and the residue was treated as in the experiment. The cultures developed equally well in all three series without exhibiting the least effect of the extract.

Lastly, an extract was prepared by centrifuging 100–150 cultures, drying the washed amoeba sediment over $CaCl_2$ at room temperature in an desiccator, and extracting with 0.1 per cent NaCl solution for 48 hours. At first these experiments, too, failed to produce any result. However, in the second part of this experiment the nutrient solution employed had been previously autoclaved four times over. Such a solution considerably prolongs the period of inhibited division at the beginning of each culture. Therefore, it was hoped that in such a culture the stimulation of division, if at all existent, should become most clearly manifest. In fact, in two out of seven cultures a marked positive divergence from the controls without extract was observed. However remarkable this effect may be, no conclusive value can be attributed to it. But it may be possible, by the same method with improved technique, to achieve a positive result with certainty in all cases.

DISCUSSION

Surveying the state of the problem once more, we must emphasize, first of all, that cultures containing bacteria will hardly yield conclusive results. The papers quoted above show clearly how the multiplication of the Protozoa in cultures is largely dependent on the bacteria present. Even in those cases where the numbers of bacteria were determined, their possible influence on the Protozoa remained undefined. If it be assumed that the allelocatalytic effect is due to substances given off by the living cell, the bacteria present may act in two possible ways: either they may give off a similar substance themselves, thus masking the effect of variation in protozoan densities, or, on the other hand, they may destroy the substance. In both cases the effect of the allelocatalytic agent will not be demonstrated by the experiment. A similar argument would hold if the effect should be due to rays (like the mitogenetic rays) instead of chemical substances. We should consider, moreover, that experiments with cultures containing bacteria are not to be reproduced without the utmost difficulty and, last but not least, that an exact analysis of the metabolism of the Protozoa which might enable an insight into the phenomenon is hardly possible in such cultures. Petersen (1929) and Johnson (1933) showed that the results vary according to whether small quantities (2 drops) or large volumes (20–40 drops) of nutrient solution are used. It must again be stressed, in this connection, that in the present experiments with *M. palestinensis*, hardly 17 μ in size, the allelocatalytic effect did not appear before there were more than 0.3 cu. mm. medium per individual. We must conclude that in a large percentage of the experiments the volumes of medium used were too small in relation to the volume of the animals present. Furthermore, the use of smallest quantities of nutrient solution involves a number of other sources of error, such as, variability in the pH, change in salt concentrations due to evaporation, and, particularly, accumulation of metabolic products, perhaps combined with scarcity of food.

We are thus left with only the experiments of Phelps (1935), who worked on mass cultures free of bacteria but whose results are directly opposed to mine. I could not detect the cause of this contradiction in his conduction of the experiments, though possibly the yeast extracts employed may have introduced allelocatalytically active substances into the medium, or the high rate of division of *Glaucoma* may have rapidly obliterated the differences existing in the initial stages, so that they did not find expression in the results.

Although my experiments confirm the allelocatalytic effect as such, they do not contribute to the explanation of the phenomenon. Robertson's hypothesis of discharge of allelocatalytically active substances from the cell during division could only be established if such substances were to be actually detected in old nutrient media or in cell extracts. Before this is achieved, the theory cannot claim higher probability than any other possible explanation. Of these, the mitogenetic radiation may be specially mentioned. As far as known to me, it has not been investigated in this connection, though it is not out of question as a possible cause of the described phenomena.

SUMMARY

The problem of allelocatalysis was investigated in mass cultures of *Mayorella palestinensis*, free of bacteria; and the following results were obtained:

1. The cultures whose initial population was less than 0.3 amoebae per 0.1 cu. mm. of solution showed a distinctly lower rate of division in the first days than the controls with higher initial populations. With increasing density of population, the rate of division in the less densely populated cultures increases, and the maximum population eventually attained is largely independent of the quantity of inoculum.
2. Neither nutrient solutions in which amoebae had been cultured for various lengths of time nor amoeba extracts succeeded in producing any marked increase in division rate.

These results, though confirming the fact of allelocatalysis, cannot afford a support to Robertson's explanation of this phenomenon.

LITERATURE CITED

ALLEE, W. C. 1931. Animal aggregations. Chicago: University of Chicago Press.

BEERS, C. D. 1933. The relation of density of population to rate of reproduction in the ciliates *Didinium nasutum* and *Stylonychia pustulata*. Arch. f. Protistenk., 80:36.

CALKINS, G. N. 1926. The biology of the Protozoa. Philadelphia: Lea & Febiger. Pp. 623.

CHEJFEC, M. 1929. Die Lebensdauer von *Paramaecium caudatum* in Abhangigkeit von der Nahrungsmenge. Acta biol. exper., 4:73.

CUTLER, D. W., and CRUMP, L. M. 1923. The rate of reproduction in artificial cultures of *Colpidium colpoda*. II. Biochem. Jour., 17:875.

———. 1924. The rate of reproduction in artificial cultures of *Colpidium colpoda*. III. *Ibid.*, 18:903.

———. 1925. The influence of washing on the reproductive rate of *Colpidium colpoda*. *Ibid.*, 19:450.

DARBY, HUGH H. 1930. Studies on growth acceleration and yeast. Jour. Exper. Biol., 7:308.

FISCHER, A. 1933. Gewebezuchtung und ihre Beziehung zur allgemeinen Biologie. Zool. Anz., 6, Suppl., p. 83.

GREENLEAF, W. E. 1926. The influence of volume of culture medium and cell proximity on the rate of reproduction of Infusoria. Jour. Exper. Zoöl., 46:143.

GRIMWALD, E. 1928. Recherches sur les facteurs du développement des cultures de microorganismes. L'Action de la substance allélocatalitique apparait-elle dans les cultures du *Colpidium colpoda* Ehrb. Acta biol. exper., 3:81.

JAHN, T. L. 1929. Studies on the physiology of the euglenoid flagellates. I. The relation of the density of population to the growth rate of *Euglena*. Biol. Bull., 57:81.

JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., 6:22.

———. 1936. Studies on the nutrition and reproduction of *Paramecium*. *Ibid.*, 9:1.

MYERS, E. C. 1927. Relation of density of population and certain other factors to survival and reproduction in different biotypes of *Paramecium caudatum*. Jour. Exper. Zoöl., 49:1.

PESKETT, G. L. 1924. Allelocatalysis and the growth of yeast. Biochem. Jour., 18:866.

———. 1925. Studies on the growth of yeast. I and II. *Ibid.*, 19:464 and 474.

PETERSEN, W. A. 1929. The relation of density of population to rate of reproduction in *Paramecium caudatum*. Physiol. Zoöl. 2:221.

PHELPS, A. 1935. Growth of Protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and of the amount of the inoculum. Jour. Exper. Zoöl., 70:1.

REICH, K. 1935. The cultivation of sterile amoeba on media without solid food. Jour. Exper. Zoöl., 69:497.

ROBERTSON, T. B. 1921. Experimental studies on cellular multiplication. Biochem. Jour., 15:595.

———. 1924*a*. Allelocatalytic effect in cultures of *Colpidium* in hay-infusion and in synthetic media. *Ibid.*, 18:1240.

———. 1924*b*. The influence of washing upon the multiplication of isolated Infusoria and upon allelocatalytic effect in cultures initially containing two Infusoria. Australian Jour. Exper. Biol. and Med. Sci., 1:151.

———. 1927. On some conditions affecting the viability of cultures of Infusoria and the occurrence of allelocatalysis therein. *Ibid.*, 4:1.

YOCUM, H. B. 1927. The effect of quantity of culture medium on the division rate of *Oxytricha*. Anat. Rec., 37:144.

Physiological Zoölogy

Vol. XI OCTOBER 1938 *No. 4*

THE EFFECT OF SUBSTANCES PRODUCED BY CHILOMONAS PARAMECIUM ON RATE OF REPRODUCTION

(Two figures)

S. O. MAST AND D. M. PACE

The Zoölogical Laboratory of The Johns Hopkins University

TABLE OF CONTENTS

Introduction 360

Material and methods 360

Relation between the number of chilomonads in a given quantity of culture fluid and the rate of reproduction 361

Relation between quantity of culture fluid and rate of reproduction 361

Relation between number of chilomonads, volume of culture fluid, and rate of reproduction 363

Factors involved in the correlation between density of population and rate of reproduction in *Chilomonas* 365

Relation between rate of reproduction and rate of growth in *Chilomonas* 366

The nature of the change in the culture fluid produced by *Chilomonas* 369

Minimum and optimum concentrations in the culture fluid, of substance produced by *Chilomonas*, as indicated by its effect on rate of reproduction 372

Relation between the concentration in the protoplasm, of substance produced by *Chilomonas* and rate of reproduction 374

Discussion 379

Summary 380

Literature 381

INTRODUCTION

WOODRUFF (1911, 1913) concludes that *Paramecium aurelia*, *P. caudatum*, *Stylonychia pustulata* and *Pleurotricha lanceolata* excrete substances which retard growth.

Robertson (1922, 1923, 1924) maintains that *Enchelys farcimen* and an "infusorian," probably *Colpidium colpoda*, produce substances which accelerate growth. Cutler and Crump (1923, 1924, 1925) repeated Robertson's experiment with *C. colpoda* but found no evidence in support of his contention, nor did the following investigators in similar experiments on various unicellular organisms: Peskett (yeast, 1924, 1925); Greenleaf (*P. caudatum*, *P. aurelia*, *Pleurotricha lanceolata*, and *Stylonychia pustulata*, 1924, 1926); Calkins (*Uroleptus mobilis*, 1926); Myers (*Paramecium caudatum*, 1927); Grimwald (*Colpidium colpoda*, 1928); Chejfec (*Paramecium caudatum*, 1929); Jahn (*Euglena*, 1929); Darby (*Paramecium caudatum*, *Paramecium aurelia*, and *Stylonychia pustulata*, 1930); Di Tomo (*Paramecium caudatum*, 1932); Beers (*Didinium nasutum* and *Stylonychia pustulata*, 1933); Johnson (*Oxytricha* sp., 1933); Phelps (*Glaucoma pyriformis*, 1935, 1936).

However, Yocom (1928) in observations on *Oxytricha* sp. and Petersen (1929) and Dimitrowa (1932) in observations on *P. caudatum*, obtained results which seem to support Robertson's contention.

Chejfec (1929) in accurately controlled experiments demonstrated that the rate of growth in *P. caudatum* is closely correlated with the concentration and the availability of bacteria in the culture medium; and he concluded that the increase in rate of growth observed by Robertson, Yocom, Petersen and Dimitrowa was probably due to increase in the concentration and the availability of the bacteria in the culture medium, not to growth-promoting substance produced by the protozoa in it. Johnson (1933) and Phelps (1935, 1936) on the basis of results obtained in extensive well-planned observations on *Oxytricha* sp. and *Glaucoma pyriformis*, came to essentially the same conclusion.

It is consequently obvious that the evidence in support of Robertson's contention, namely, that protozoa produce a growth-promoting substance, is very much less extensive than that in opposition to it, and that, to obtain worth-while results concerning this contention, variation in the quality, the quantity and the availability of food, as well as variation in other environmental factors, must be accurately controlled.

We have done this in the experiments on *Chilomonas paramecium* considered in the following pages. This organism grows well in sterile solutions which contain only relatively simple chemicals in known concentrations. It is therefore well suited for experiments concerning the problem under consideration.

MATERIAL AND METHODS

The chilomonads used in these experiments were obtained from a culture which had been grown in our laboratory for nearly six years in a sterile solution consisting of $NaC_2H_3O_2$, 150 mg; NH_4Cl, 46 mg; $(NH_4)_2SO_4$, 10 mg; K_2HPO_4, 20 mg; $CaCl_2$, 1 mg; $MgCl_2$, 1 mg; and H_2O, 100 cc, with slight modifications (Mast and Pace, 1933, 1935, 1937; Mast, Pace, and Mast, 1936).[1] This solution will be referred to as the acetate-ammonium culture fluid. The salts in it were Kahlbaum's purest and the water was triple (pyrex glass) distilled (Mast, 1928). Several different methods were used. These will be described in detail in the different sections in the following pages. The cultures were sterile thruout in all, and pyrex glass was used thruout in all, in accord with earlier work (Mast and Pace, 1933, p. 328).[2]

---

[1] We found that mass cultures in this solution at room temperature die out in 6–10 days but that if some are transferred to fresh culture fluid every second or third day, they live indefinitely.

RELATION BETWEEN THE NUMBER OF CHILOMONADS IN A GIVEN QUANTITY OF CULTURE FLUID AND THE RATE OF REPRODUCTION

About 50 chilomonads, taken from a vigorous culture growing in sterile acetate-ammonium culture fluid, were put into 0.4 cc fresh sterile culture fluid in a depression on a slide and left 2 or 3 days. Numerous specimens were taken from this culture and passed successively thru 0.4 cc fresh sterile culture fluid in each of 4 depressions. Then 0.4 cc fresh sterile culture fluid was put into each of 16 depressions on sterile slides in petri dishes, and one of the prepared chilomonads into each of 4 of the depressions, 5 into each of 4, 25 into each of 4, and 50 into each of 4, after which all the slides were put into a water-bath at 24.4 ± 0.2° C. and left 24 hours. Then the chilomonads in each of the depressions were counted and the number recorded. This whole process was repeated eight times. The results obtained are presented in table 1.

This table shows that there were produced in 24 hours, for each chilomonad introduced, an average of 8.75 chilomonads in the depressions which had received 1 chilomonad, 10.50 in those which had received 5 chilomonads, 12.42 in those which had received 25 chilomonads, and 13.78 in those which had received 50 chilomonads. That is that an increase from one chilomonad to 50 in 0.4 cc culture fluid resulted in an increase in the rate of reproduction of 50.6 percent.

This seems to prove conclusively that, within certain limits, the rate of reproduction of *Chilomonas* varies directly with the density of the population. If this is true, then a given number of specimens in a small volume of culture fluid should reproduce more rapidly than the same number in a large volume. This proposition was tested in the following experiments.

RELATION BETWEEN QUANTITY OF CULTURE FLUID AND RATE OF REPRODUCTION

Experiment 1.—Chilomonads were prepared as described above. Then 0.4 cc culture fluid was put into each of 4 depressions on slides in petri dishes, and one of the prepared chilomonads was added to the solution in each depression. This was then repeated with six other quantities of culture fluid. All were then kept in a water-bath at 24.4 ± 0.2° C. for 24 hours, after which the chilomonads were counted. The whole process, with the exception of a small variation in temperature, was then repeated twelve times. The results obtained are presented in table 2.

This table shows that, as the volume in the depressions decreased, the average number of chilomonads produced in 24 hours from each one introduced, increased from 9.15 in 0.4 cc to 12.98 in 0.006 cc, i.e., more than 40 percent.

---

[2] A few of our cultures occasionally became turbid with bacteria or infected with mold, which could readily be seen. These were invariably discarded. Some of the others were, from time to time, tested for bacteria by plating on standard nutrient agar. With the exception of an occasional colony or two, we found no indication of bacteria. It is, however, well known that some forms do not grow on this medium. Dr. William T. Burrows, professor of bacteriology in the University of Chicago, made in our laboratory various tests, microscopic as well as cultural, for such forms in our cultures, but found none. It can therefore be concluded that they were actually sterile. An account of these tests will soon appear in *Protoplasma.*

These results therefore strongly support the conclusion reached in the preceding experiment. This conclusion is also supported by the results obtained in the following experiment, in which observations were made on the time at which division in chilomonads in different volumes of culture fluid occurs.

Experiment 2.—One-tenth cc culture fluid was put into each of 12 depressions on slides, and 0.006 cc into each of 12. Then one chilomonad, prepared as described above, was added to the solution in each depression. All the slides were put into a water-bath at 24.4 ± 0.2° C., after which the chilomonads in each depression were counted every half-hour for 6 hours. This was then repeated with other chilomonads. The results obtained are presented in table 3.

TABLE 1

RELATION BETWEEN NUMBER OF CHILOMONADS IN A GIVEN VOLUME OF CULTURE FLUID AND FREQUENCY OF DIVISION

Each day the chilomonads put into all the depressions were taken from those produced the preceding day in depressions 9, 10, 11, or 12. 1st–9th, days; hydrogen-ion concentration, pH 6.8; temperature, 24.4±0.2° C.; Chi., chilomonads.

No. of Chi. Put into Each Depression	Designation of Depressions	Average Number Produced in 24 Hours from Each Chi. Introduced									Total Average	σ
		1st	2d	3d	4th	5th	6th	7th	8th	9th		
1....	1	11.00	8.00	11.00	9.00	8.00	5.00	8.00	6.00	11.00	8.75±0.27	2.45
	2	8.00	12.00	12.00	13.00	5.00	5.00	14.00	8.00	8.00		
	3	10.00	11.00	7.00	11.00	8.00	8.00	8.00	6.00	6.00		
	4	6.00	8.00	12.00	7.00	8.00	6.00	10.00	8.00	13.00		
5....	5	10.40	8.00	8.80	16.80	7.40	12.20	13.60	8.60	9.40	10.50±0.44	3.98
	6	9.00	13.40	13.00	14.60	8.60	7.40	8.80	7.80	14.40		
	7	11.60	6.40	11.20	10.80	9.00	11.80	13.20	8.20	10.60		
	8	12.60	9.00	11.60	12.60	11.60	8.40	7.40	10.20	9.60		
25....	9	10.96	13.08	12.04	14.92	11.12	14.28	12.32	10.16	13.60	12.42±0.23	2.06
	10	11.48	11.88	14.76	14.84	11.44	13.48	11.68	13.92	15.00		
	11	11.16	13.32	10.48	15.56	12.16	14.08	8.08	8.88	14.08		
	12	9.12	14.56	12.72	16.32	11.24	12.88	9.68	9.40	12.68		
50....	13	......	......	......	19.84	11.74	11.52	14.48	14.24	19.68	13.78±0.38	2.83
	14	......	......	......	15.70	10.48	12.44	14.92	11.28	16.46		
	15	......	......	......	13.88	13.78	13.96	7.06	10.96	15.92		
	16	......	......	......	17.22	10.72	11.88	13.08	15.12	13.68		

This table shows that in the first test, 2 of the 12 chilomonads, each in 0.006 cc culture fluid, had divided in 2 hours, and none of the 12, each in 0.1 cc culture fluid; that in 3 hours, 7 of the former had divided and only 2 of the latter; and that in 6 hours the former produced 33 chilomonads and the latter only 24. It shows also that in the second test the results obtained were essentially the same as in the first.

It can therefore be concluded that the rate of reproduction in *Chilomonas* varies inversely with the volume of culture fluid per individual. The following experiment concerns the extent of this correlation.

RELATION BETWEEN NUMBER OF CHILOMONADS, VOLUME OF CULTURE FLUID AND RATE OF REPRODUCTION

Chilomonads were prepared as in the preceding experiment. Then 0.1 cc culture fluid was put into each of 20 depressions on slides in petri dishes, and 1 chilomonad added to each of 4 depressions, 5 to each of 4, 10 to each of 4, 25 to each of 4, and 50 to each of 4. All were then put into the water-bath at 24.4 ± 0.2° C. This was repeated three times, once with 0.05 cc culture fluid in each depression, once with 0.025 cc and once with 0.0125 cc. After 24 hours the chilomonads in all the depressions were counted. The whole process was then repeated twelve times. The results are presented in table 4.

TABLE 2

RELATION BETWEEN VOLUME OF CULTURE FLUID AND FREQUENCY OF DIVISION IN *Chilomonas*

Each day the chilomonads put into the depressions were taken from those produced the preceding day in depressions 1, 2, 3, or 4. 1st–13th, days; hydrogen-ion concentration, pH 6.8; temperature, 24.4±0.2° C. first to 6th days, 24.7° C. 7th to 10th days.

Volume of Culture Fluid (cc)	Designation of Cultures	Number Produced in 24 Hours from Each Chilomonad Introduced													Total Average
		1st	2d	3d	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	
0 006	1	10	12	16	8	14	8	8	16	16	12	14	12	8	12.98
	2	12	16	8	10	8	8	15	16	16	16	8	8	14	
	3	8	14	8	8	18	14	30	22	8	8	9	14	16	
	4	16	9	8	16	8	8	16	16	31	14	30	8	8	
0.012	5	9	10	8	8	8	9	16	15	10	8	12	8	13	11 38
	6	16	16	8	7	12	8	15	16	15	10	12	8	16	
	7	8	8	12	8	8	6	10	8	25	16	10	11	8	
	8	10	12	8	15	10	16	24	12	16	7	7	6	8	
0.025	9	12	12	13	6	8	8	16	16	15	7	8	10	11	10.04
	10	8	16	8	8	14	5	15	8	6	16	16	5	4	
	11	10	14	4	8	8	8	8	16	15	13	8	8	10	
	12	8	10	8	12	8	8	14	8	8	8	12	8	9	
0.05	13	11	10	14	13	8	4	16	8	15	8	8	8	14	10.61
	14	10	8	4	7	7	8	6	16	16	12	8	8	8	
	15	9	12	8	10	8	8	8	8	12	6	16	4	8	
	16	8	8	12	8	10	8	8	16	8	10	8	8	8	
0.1	17	12	8	16	8	11	8	14	16	16	15	7	16	8	10.54
	18	9	8	8	7	7	16	10	12	10	14	16	12	8	
	19	8	12	4	14	8	8	8	8	15	8	8	8	7	
	20	10	14	7	6	8	8	13	10	14	12	22	8	8	
0.2	21	9	8	8	8	8	12	15	14	15	14	8	8	8	9.69
	22	8	10	8	8	14	8	14	16	8	8	14	8	7	
	23	10	8	9	8	8	8	12	8	16	8	8	14	8	
	24	7	8	8	10	8	6	10	10	14	8	8	8	8	
0.4	25	8	8	4	14	7	8	15	16	11	15	8	8	8	9.15
	26	8	7	8	8	12	6	8	18	16	7	12	4	4	
	27	12	6	11	8	8	7	9	8	12	8	7	8	12	
	28	8	8	10	4	4	11	14	16	10	7	8	12	8	

By referring to table 4 it will at once be seen that in all the volumes tested, except the lowest, as the number of chilomonads introduced increased, the number produced in 24 hours, per individual introduced, increased to a maximum and then decreased; and that the optimum for the number introduced was about 25 for 0.1 cc culture fluid, about 10 for 0.05 cc, and about 7 for 0.025 cc, i.e. that the optimum number varies directly with the volume.

TABLE 3

RELATION BETWEEN VOLUME OF CULTURE FLUID AND RATE OF REPRODUCTION IN *Chilomonas*

One chilomonad was put into the solution in each depression. Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads.

VOLUME OF CULTURE FLUID IN EACH DEPRESSION (CC)	NO. OF DEPRESSIONS	TIME (IN HOURS) THE CHI. WERE IN THE CULTURE FLUID									
		0	2	2.5	3	3.5	4	4.5	5	5.5	6
		Total No. of Chi. in the Depressions									
0.1	12	12	12	12	14	16	19	21	21	23	24
0.006.	12	12	14	16	19	20	23	24	24	25	33
0.1......... ...	12	12	......	.....	13	....	17	17	19	22	24
0.006	12	12	...	......	17	......	18	22	24	26	31

TABLE 4

RELATION BETWEEN VOLUME OF CULTURE FLUID, NUMBER OF CHILOMONADS, AND RATE OF REPRODUCTION

Each figure represents the average number of chilomonads produced in 24 hours from each one introduced (1 in each of 4 depressions, 5 in each of 4 depressions, etc., on 13 different days). Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads.

NO. OF CHI. PUT INTO EACH DEPRESSION	VOLUME OF CULTURE FLUID IN EACH DEPRESSION (CC)			
	0.1	0.05	0.025	0.012
	Average No. of Chi. Produced from Each One Introduced			
1..............	8.89	9.08	9.67	12.19
5..............	10.12	13.18	13.72	9.20
10..............	12.28	15.10	15.12	8.80
25..............	13.63	12.58	10.50	7.41
50..............	10.34	8.88	7.76	3.76

If each volume used is divided by the optimum number, so as to obtain the optimum volume per individual, there is obtained 0.004 cc for 0.1 cc volume used, 0.005 cc for 0.05

cc volume used and 0.0036 cc for 0.025 cc volume used. This indicates that the optimum volume of culture fluid for reproduction is approximately 0.004 cc per individual.

The fact that the rate of reproduction increases to maximum as the density of population increases and then decreases, shows that for every number of individuals per given volume below the optimum number there is a number above the optimum which has the same effect on rate of reproduction. This is clearly shown by the results presented in table 4. These show, e.g. that the rate of reproduction with 1 chilomonad in 0.05 cc culture fluid is practically the same as it is with 50, but much higher with 10. If, then, one finds that the rate of reproduction is the same with a small number of organisms in a given volume of solution as it is with a larger number, it obviously does not prove that the rate of reproduction is not correlated with the density of population.

TABLE 5

EFFECT OF CHANGES PRODUCED BY *Chilomonas* IN THE CULTURE MEDIUM ON RATE OF REPRODUCTION

Temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads

DESIGNATION OF EXPERIMENT	NO. OF DEPRESSION CULTURES	VOLUME (IN CC) OF CULTURE FLUID IN EACH	NO OF CHI. PUT INTO EACH	TIME (IN HOURS) LEFT	VOLUME (IN CC) OF SOLUTION TAKEN FROM EACH	NO. OF CHI. ADDED TO EACH VOLUME	TOTAL NO. OF CHI. FOUND—							
							After Following Hours:							After 22 hr. per Chi. Introduced
							0	2	3	4	5	6	22	
A.	24	0 025	0	2	0 025	1	24	24	26	26	29	33	234	9 75
	24	0 025	5	2	0 025	1	24	26	27	28	39	43	304	12 75
B......	8	0 1	0	16 5	0.025	1	8	8	8	8	10	10	104	13.00
	8	0.1	1	16.5	0.025	1	8	8	10	12	13	15	117	14.62
	8	0.1	10	16 5	0 025	1	8	10	12	14	14	17	108	13.50
	8	0.1	50	16 5	0.025	1	8	8	8	9	10	13	68	8.50
	8	0 1	200	16 5	0.025	1	8	8	8	9	10	12	48	6.00
C......	12	0.1	0	16.5	0.025	1	12	12	14	14	16	16	125	10.41
	11	0.1	1	16.5	0.025	1	11	14	14	15	18	18	150	13.33
	12	0.1	10	16 5	0.025	1	12	15	17	18	20	21	128	10.66
	12	0.1	25	16.5	0.025	1	12	16	17	20	21	22	103	8.58
	8	0.1	400	16.5	0.025	1	8	8	6	5	1	0	0	0

The question now arises as to what causes the observed correlation between volume of culture per individual or density of population and rate of reproduction.

FACTORS INVOLVED IN THE CORRELATION BETWEEN DENSITY OF POPULATION AND RATE OF REPRODUCTION IN *Chilomonas*

One immediately thinks of changes in the chemical composition of the culture fluid, owing to substances produced or consumed by the organisms, as the cause of the observed correlation between density of population and rate of reproduction; but it is obvious that other factors may be involved, e.g. contact stimulation due to movement of the organisms or mitogenetic rays produced by them.

If it is due to changes in the chemical composition of the culture fluid, the rate of reproduction in culture fluid in which chilomonads have been for some time should differ from that in fresh culture fluid. The following experiments concern this contention. These experiments were performed as follows:

Different numbers of sterile chilomonads were put into different quantities of culture fluid and left different periods of time; then all the chilomonads were removed, and a given amount of the culture fluid was put into each of a number of depressions on slides and one chilomonad (prepared as described above) added to each; then the slides were put into a water-bath at 24.4 ± 0.2° C. and the chilomonads counted at definite intervals. The results obtained are presented in table 5.

This table shows that three experiments were made and that in all reproduction under optimum conditions began markedly sooner and continued at a much higher rate in the culture fluid in which chilomonads had been for some time than in fresh culture fluid. It shows also that as the number of chilomonads which had been in the culture fluid increased, the rate of reproduction increased to a maximum and then decreased to zero. It shows, moreover, that to produce a favorable effect on the rate of reproduction requires a surprisingly short time and that fission is not necessary. This is clearly shown in the results obtained in Experiment A, in which only 5 individuals had been in the culture fluid only 2 hours and none had divided.

Since the differences in rate of reproduction recorded in table 5 were independent of the immediate density of the population in the culture fluid, it is evident that they must have been due to changes in the composition of the culture fluid produced by the chilomonads which had been in it before the records were begun. The results presented in this table therefore prove that *Chilomonas* produces changes in the composition of the culture fluid which promote reproduction if they are moderate and inhibit reproduction if they are excessive; but they do not show whether these changes are due to production or consumption of substances, or both. This problem is considered in the following pages; but before taking it up, we will consider the relation between rate of reproduction and rate of growth under the conditions which obtained in the preceding experiments.

RELATION BETWEEN RATE OF REPRODUCTION AND RATE OF GROWTH IN *Chilomonas*

Under some conditions the rate of reproduction in *Chilomonas* is not closely correlated with rate of growth; e.g. high concentration of carbon dioxide inhibits rate of reproduction much more effectively than rate of growth. This results in very large specimens (Mast and Pace, 1933). The question now arises as to whether or not the changes in the rate of reproduction caused by changes in the culture fluid produced by the action of *Chilomonas* are accompanied by corresponding changes in the rate of growth. If they are, the structure and the size of chilomonads grown in culture fluid which has been acted on by them should be essentially the same as the structure and the size of those grown in fresh culture fluid. This proposition was investigated as follows:

Into each of 12 depressions on slides in petri dishes 0.1 cc culture fluid was put; then 75 chilomonads, taken from a flourishing culture, were added to the solution in each of 4 of the depressions; 400, taken from the same culture, to that in each of 4 others; and none to that in the remaining 4. The petri dishes were then put into the water-bath at 24.4 ± 0.2° C. and left 4 hours. All the chilomonads were then removed, and the solution in the 4 depressions in each of the three groups mixed, respectively. Then 0.025 cc of one of the three mixtures was put into each of 12 depressions in petri dishes; 0.025 cc of another one of the mixtures into each of 12; and 0.025 cc of the remaining mixture into each of 12. A small chilomonad (produced by recent division) from a flourishing culture, called the "parent culture," was now added to the solution in each of the 36 depressions, and all put into the water-bath at 24.4 ± 0.2° C., after which the chilomonads in each were counted every hour for 6 hours. Then all the chilomonads in the 36 depressions and 10 taken from the parent culture were fixed and stained for starch and fat, first in Lugol solution and then in Sudan III and sketched (Fig. 1). The 10 chilomonads taken from the parent culture were, in size and in other respects, like those put into the culture fluid in the 36 depressions at the beginning of the experiment.

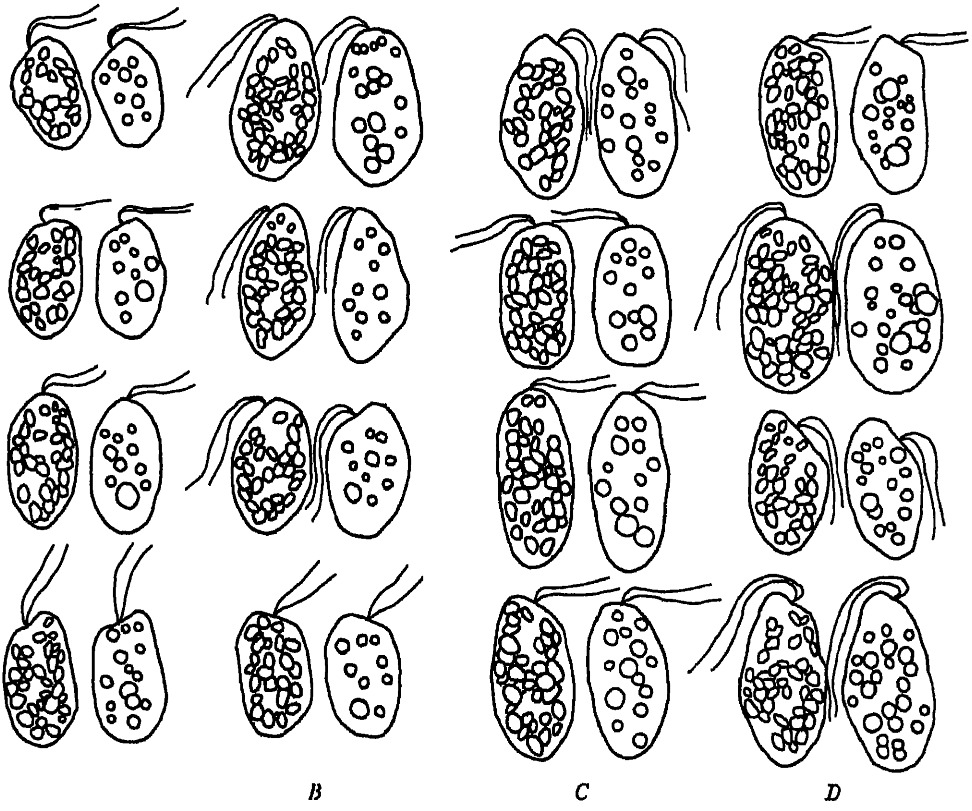


FIG. 1.—Camera outlines showing the effect produced on size and on starch and fat content in *Chilomonas* by changes produced in the culture fluid by it. *A*, small specimens used to start three depression-slide cultures—*B*, *C*, and *D*. *B*, chilomonads taken at random from 7-hour-old cultures in fresh acetate-ammonium culture fluid; *C*, chilomonads taken at random from 7-hour-old cultures in acetate-ammonium culture fluid in which 75 chilomonads per 0.1 cc had been for 4 hours; *D*, chilomonads taken at random from 7-hour-old cultures in acetate-ammonium culture fluid in which 400 chilomonads per 0.1 cc had been for 4 hours. Each pair of large outlines consists of two made of the same specimen. The small outlines in the large outline at the left represent starch grains; those in that at the right represent fat droplets. Hydrogen-ion concentration, pH 6.8; temperature, 24.4 ± 0.2° C.

In the chilomonads in the fresh culture fluid, the first division occurred during the fourth hour in it (Table 6); in those in the culture fluid in which 75 chilomonads had been for 4 hours, it occurred during the second hour; and in those in the culture fluid in which 400 chilomonads had been for 4 hours, it did not occur until the sixth hour.

In the first of these solutions 8 of the 12 specimens divided in 7 hours; in the second, 12; and in the third, 7. It is consequently evident that in the culture fluid in which 75 chilomonads had been for 4 hours, fission began markedly sooner and continued at shorter intervals than in the fresh culture fluid; and that in the culture fluid in which 400 chilomonads had been for 4 hours, it began considerably later and continued at longer intervals than in the fresh culture fluid. There was, then, a very definite difference in the rate of reproduction in these culture fluids.

If, now, the sketches (Fig. 1) of specimens taken from them are compared, it will be seen that there was no essential difference in the starch and the fat content, but that those from the culture fluids which had had respectively 75 and 400 chilomonads were somewhat larger than those from the fresh culture fluid, and much larger than those taken from the parent culture.

RELATION BETWEEN INCREASE IN RATE OF REPRODUCTION AND INCREASE IN RATE OF GROWTH CAUSED BY CHANGES IN THE CULTURE FLUID PRODUCED BY *Chilomonas*

Temperature, 24.4±0.2° C; hydrogen-ion concentration, pH 6.8, "ac-am," acetate-ammonium culture fluid; Chi., chilomonads.

CULTURE FLUID USED	TOTAL NO. OF CHI. IN 12 DEPRESSION CULTURES AFTER FOLLOWING HOURS					VOLUME IN Cμ OF CHI. PRODUCED IN 7 HR.	
	0	4	5	6	7	Total	Av. per Individual
Fresh ac-am .	12	13	15	18	20	46,700	2,333
Ac-am in which 75 chi. per 0.1 cc had been 4 hr. . .	12	17	18	23	24	76,872	3,202
Ac-am in which 400 chi. per 0.1 cc had been 4 hr. .	12	12	12	17	19	53,048	2,792
Volume of 12 chi. used to start cultures .						17,040	1,494

The volume of each of the chilomonads sketched was calculated by means of the length and the width obtained from measurements made on the sketches and on the assumption that they are ellipsoidal in form.[3] The results obtained are presented in table 6. These results indicate that the total volume of the chilomonads produced in the 12 depressions which contained fresh culture fluid was 46,700 cμ (an average of 2,333 cμ per chilomonad), that the total volume of those produced in the culture fluid which had had 75 chilomonads for 4 hours was 76,872 cμ (an average of 3,202 cμ per chilomonad), that the total volume of those produced in the culture fluid which had had 400 chilomonads for 4 hours was 53,048 cμ (an average of 2,792 cμ per chilomonad), and that the volume of those taken from the parent culture to start each of the three sets of cultures referred to above was 13,940 cμ (an average of only 1,494 cμ per chilomonad).

---

[3] *Chilomonas* is slightly flattened. The camera outlines show the maximum width. This width was used in calculating the volumes presented. These are, therefore, somewhat too large.

It is consequently evident that the average volume per chilomonad increased considerably in all three culture fluids, i.e. from 1,494 cμ to 2,333, 3,202, and 2,792 cμ, respectively. The fact that it increased more in the culture fluid in which 75 chilomonads had been for 4 hours than in the fresh culture fluid shows that the change produced by the 75 chilomonads in the culture fluid caused, not only an increase in the rate of reproduction, but also an increase in the rate of growth and that the increase in the rate of growth was, in fact, considerably greater than the increase in the rate of reproduction. It can consequently be concluded that the change in the culture fluid produced by *Chilomonas* acts primarily on the rate of growth and secondarily on the rate of reproduction.

THE NATURE OF THE CHANGE IN THE CULTURE FLUID PRODUCED BY *Chilomonas*

About 1,000 sterile chilomonads were put into 1.5 cc sterile culture fluid in a columbia dish in a petri dish. These multiplied and soon became very abundant but all died in about 6 days. Then some of the solution in the dish was put into each of five small pyrex glass tubes and the same amount of fresh culture fluid into each of two. One of the former and one of the latter were left at room temperature. All the rest were suspended in water in a beaker over a gas flame and left until it began to boil; then one of those containing old culture fluid was removed, 30 minutes later another of these and the one containing fresh culture fluid, 30 minutes later another containing old culture fluid and 30 minutes later still another. After the tubes had cooled to room temperature, 0.025 cc of the solution in each of these tubes and those left at room temperature was put, respectively, into each of 12 depressions on slides in petri dishes; then a small chilomonad from a vigorous culture was passed through four separate portions of fresh culture fluid and added to each, after which all were put into the water-bath at 24.2 ± 0.2° C. and examined from time to time for 22 hours. The results obtained are presented in table 7. This experiment was repeated. The results obtained are practically the same as those presented.

Table 7 shows that there were produced in the 12 depressions with fresh culture fluid, not heated, 8 individuals in 8 hours and 142 in 22 hours and practically the same number in the fresh culture after it had been at 100° 30 minutes; and that in the old culture fluid, not heated, none were produced in 8 hours and only 34 in 22 hours, but that in this fluid after it had been at 100° for 60 minutes there were produced 9 in 8 hours and 140 in 22 hours, i.e. very nearly the same number as in the fresh culture fluid.

The fact that subjecting the old culture fluid to 100° for 60 minutes caused an increase in the rate of reproduction from nearly zero to practically that which obtains in fresh culture fluid demonstrates conclusively that the reduction in rate of reproduction with increase in the age of the culture is not due to consumption of substances by the organisms in the culture fluid and that it must therefore be due to the production of substance by them; and it demonstrates, moreover, that the substance produced is heat-labile. The results presented have, however, no direct bearing on the nature of the changes produced by the organisms in the culture fluid, which cause an increase in the rate of reproduction. The following experiments concern this problem.

These experiments were performed in essentially the same way as the preceding except that, in place of leaving the chilomonads in the culture fluid until the culture died out, they were left only 4 hours. But in some of these experiments there were 35 chilomonads per 0.1 cc acetate-ammonium culture fluid and in others, 150. The results obtained in the former are presented in table 8, and those obtained in the latter in table 9.

Table 8 shows that the average time required for division in fresh acetate-ammonium culture fluid was, in the two experiments made, respectively 6.75 and 6.83 hours, and that subjecting this fluid to 100° for 30 minutes did not significantly change the time required; but that, after 35 chilomonads per 0.1 cc had been in this fluid 4 hours, the average time was reduced to 6.16 and 6.33 hours, respectively, and that, as the time this fluid was subjected to 100° increased, the average time for division increased, reaching, after 30 minutes at 100°, respectively 6.91 and 6.75 hours in the two experiments, i.e. practically the same as in the fresh fluid.

THE EFFECT OF TEMPERATURE ON THE MAXIMUM CHANGE PRODUCED BY *Chilomonas* IN CULTURE FLUID

"Ac-am," acetate-ammonium culture fluid; "old ac-am fluid," culture fluid in which a culture of chilomonads had been until it died out; Chi., chilomonads; temperature, 24.2 ±0.2° C.; hydrogen-ion concentration, pH 6.8.

CULTURE FLUID USED	DEPRESSION CULTURES											
	No. of Cultures	Quantity of Fluid (cc) in Each	No. of Chi. Added to Each	Total No. of Chi. after Following Hours:							Total No. per Individual Introduced	Time (in hours) to First Division
				1	4	5	6	7	8	22		
Fresh ac-am fluid at room temperature .	12	0 025	1	12	12	13	16	18	20	154	12 83	7 41
Fresh ac-am fluid after having been at 100° for 30 min.........	12	0.025	1	12	12	13	15	18	21	155	12.91	7 41
Old ac-am fluid at room temperature.....	12	0.025	1	12	12	12	12	12	12	46	3 83	No division in 8 hr.
Old ac-am fluid after having been at 100° for 10 sec. ...	12	0.025	1	12	12	12	13	15	18	118	9.83	7.88
Same, except at 100° for 30 min...	12	0.025	1	12	12	15	19	20	24	142	11.83	6 50
Same, except at 100° for 60 min.........	12	0.025	1	12	12	14	15	18	21	152	12.66	7.33
Same, except at 100° for 90 min.........	12	0.025	1	12	12	13	15	17	20	150	12 50	7.58

This demonstrates conclusively that the change in the culture fluid produced by the chilomonads, which accelerates rate of reproduction, is not due to consumption of anything in the fluid by the chilomonads and that it therefore must be due to a substance produced by them. The fact that temperature destroys this substance just as it does that which inhibits reproduction, indicates that these substances have the same properties; and the fact that if chilomonads are left in the culture fluid only a short time, the rate of reproduction is accelerated, whereas if they are left a long time it is inhibited, indicates that the effect of the substance produced by *Chilomonas* depends upon the concentration, a weak solution causing acceleration and a strong solution inhibition.

These conclusions are supported by the fact that it requires much longer exposure to high temperature to destroy the inhibiting substance than the accelerating, as will be clearly seen by comparing tables 7 and 8. They are also supported by the results presented in table 9.

TABLE 8

THE EFFECT OF TEMPERATURE ON SMALL CHANGES PRODUCED BY *Chilomonas* ON CULTURE FLUID

"Ac-am," acetate-ammonium culture fluid; "young ac-am fluid," culture fluid in which 35 chilomonads per 0.1 cc had been for 4 hours; temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8.

CULTURE FLUID USED	AVERAGE TIME (IN HOURS) FOR 12, EACH IN 0.025 CC FLUID			
	To First Division	First to Second Division	To First Division	First to Second Division
	Experiment A		Experiment B	
Fresh ac-am fluid at room temperature	6.75	6.18	6.83	5.12
Same, except at 100° for 15 min.	7.08	6.00	6.75	5.08
Young ac-am fluid at room temperature	6.16	4.87	6.33	4.75
Same, except at 100° for 10 sec.	6.33	5.50	6.41	5.08
Same, except at 100° for 15 min.	6.41	5.83	6.66	5.20
Same, except at 100° for 30 min.	6.91	5.64	6.75	5.25
Same, except at 100° for 60 min.	6.66	5.45	6.66	5.20

This table shows that after 150 chilomonads per 0.1 cc had been in the culture fluid for 4 hours there was, in both experiments made, an increase in the time required for division, i.e. from 5.75 to 6.00 hours in the one and from 6.50 to 7.15 hours in the other, but that after this fluid had been subjected to 100° the time required for division decreased to a minimum (as the time at 100° increased) and then increased to normal.

This indicates that after 150 chilomonads per 0.1 cc have been in the culture fluid 4 hours the substance produced by them is sufficiently concentrated to cause retardation in the rate of reproduction; whereas after only 35 per 0.1 cc have been in the fluid for 4 hours (Table 8) it is so weak that it causes acceleration, that the marked increase in the rate of reproduction after the fluid has been subjected to 100° is due to destruction of a portion of the substance and consequent decrease in concentration and that the increase in the rate of reproduction to normal after longer subjection to 100° is due to destruction of all of the substance.

The results presented show then that if culture fluid in which *Chilomonas* has died out and in which the concentration of X is maximum is subjected to temperature of 100° and then cooled, the rate of reproduction in it increases from zero to maximum, as the time at 100° increases, and then decreases to that which obtains in fresh culture fluid. This indicates that X at 100° gradually decreases from lethal concentration thru optimum concentration to zero, as the time increases.

The conclusions reached are also supported by the results obtained with old culture fluid (fluid in which the chilomonads had died out) mixed with fresh culture fluid. The methods used in the experiment made with this mixture were the same as those used in the preceding experiments. The average time required for division of 12 chilomonads, each in 0.025 cc culture fluid, was 6.83 hours for fresh fluid, more than 9 hours for old fluid, 6.83 hours for a mixture of old fluid (3 parts) and fresh fluid (1 part), 5.25 hours for old fluid (1 part) and fresh fluid (1 part), and 6.33 hours for old fluid (1 part) and fresh fluid (3 parts).

TABLE 9

EFFECT OF TEMPERATURE ON MEDIUM CHANGES PRODUCED BY *Chilomonas* ON CULTURE FLUID

"Ac-am," acetate-ammonium culture fluid; "medium ac-am fluid," culture fluid in which 150 chilomonads per 0.1 cc had been for 4 hours; temperature, 24.4±0.2° C.; hydrogen-ion concentration, pH 6.8.

CULTURE FLUID USED	AVERAGE TIME (IN HOURS) FOR 12 CHI., EACH IN 0.025 CC FLUID			
	To First Division	First to Second Division	To First Division	First to Second Division
	Experiment A		Experiment B	
Fresh ac-am fluid at room temperature	5.75	4.96	6.50	6.00
Fresh ac-am fluid after having been at 100° for 15 min	5.83	4.83	6.33	6.12
Medium ac-am fluid at room temperature	6.00	5.83	7.16	6.77
Same, except at 100° for 10 sec	4.91	4.75	5.58	6.00
Same, except at 100° for 15 min	4.25	4.75	5.25	5.58
Same, except at 100° for 30 min	4.75	4.50	5.91	5.62
Same, except at 100° for 60 min	5.75	4.75	6.41	6.12

This demonstrates conclusively that the effect on rate of reproduction of the substance produced by *Chilomonas* is specifically correlated with its concentration, the lower concentrations causing acceleration and the higher retardation.

In the following pages we shall call this substance "X."

MINIMUM AND OPTIMUM CONCENTRATIONS IN THE CULTURE FLUID OF SUBSTANCE (X) PRODUCED BY *Chilomonas*, AS INDICATED BY ITS EFFECT ON RATE OF REPRODUCTION

In ascertaining the minimum and the optimum concentrations of substance X in its effect on the rate of reproduction in *Chilomonas*, different numbers of chilomonads from a vigorous culture were put, respectively, into 0.1 cc acetate-ammonium culture fluid in depressions on slides and left 4 hours, after which all were removed. Then the rate of reproduction in the fluid in the different depressions was ascertained, as described in the preceding sections. This experiment was repeated twice. The results obtained are presented in table 10.

This table shows that, as the number of chilomonads per 0.1 cc culture fluid increased, the average time required for division of 12 specimens decreased from 6.6 hours in fresh culture fluid to a minimum of 4.87 in culture fluid in which 75 chilomonads per 0.1 cc had been for 4 hours and then increased to 7.36 hours; and that there was a slight reduction in the culture fluid in which only 1 chilomonad had been for 4 hours. It shows however that this reduction occurred in only one of the two experiments made. But in two additional experiments the average time required for the first divisions was, respectively, 8.25 and 6.83 hours in fresh culture fluid and 7.41 and 6.66 hours in culture fluid in which 1 chilomonad had been for 4 hours. There was consequently a definite reduction in three of the four tests made.

TABLE 10

THE MINIMUM AND THE OPTIMUM CONCENTRATIONS OF SUBSTANCE X IN THE CULTURE FLUID, AS INDICATED BY ITS EFFECT ON THE RATE OF REPRODUCTION IN *Chilomonas paramecium*

"Ac-am," acetate-ammonium culture fluid; Chi., chilomonads; temperature, 24.4 ± 0.2° C.; hydrogen-ion concentration, pH 6.8.

Culture Fluid Used	Time (in hours) to First Division			
	Average for 12 Chi., Each in 0.025 cc Fluid in Each of the Following Experiments:			Total Average
	A	B	C	
Fresh ac-am fluid	7.16	6.16	6.66	6.66
Ac-am fluid in which 1 chi. per 0.1 cc had been for 4 hr.	7.16		6.00	6.58
Same, except 10 chi.	6.66	5.66		6.16
Same, except 20 chi.	6.41	5.75		6.08
Same, except 50 chi.	6.41	5.41		5.91
Same, except 75 chi.		5.08	4.66	4.87
Same, except 100 chi.	5.66	5.25	5.75	5.55
Same, except 150 chi.		5.50		5.50
Same, except 200 chi.	7.00	6.75	8.33	7.36

It can therefore be concluded that the concentration of substance X produced by 1 chilomonad in 0.1 cc culture in 4 hours is high enough to have a slight accelerating effect on the rate of reproduction, that the concentration of this substance produced by 75 chilomonads in 0.1 cc culture fluid for 4 hours is optimum in its effect on rate of reproduction and that the concentration of 200 chilomonads in 0.1 cc for 4 hours causes retardation in the rate of reproduction.

If it is assumed that a chilomonad produces in 4 hours, substance X equal in amount to one-tenth its volume (which would seem to be a very liberal estimate) the concentration of this substance in the culture fluids used can readily be calculated.

The average volume of each chilomonad used was approximately 1,230 cμ. Each chilomonad would then produce approximately 123 cμ in 4 hours. The volume of the culture fluid in which substance X was produced equals 10^{11} cμ. The concentration of substance X in the culture fluid in which 1 chilomonad had been for 4 hours (i.e. the minimum effective concentration) would therefore be 123 parts of X in 10^{11} parts of culture fluid, or approximately 1 part in 800,000,000; the concentration of this substance in the culture fluid in which 75 chilomonads had been for 4 hours (i.e. the optimum concentration) would be about 1 part in 10,000,000; and the concentration of the substance in the culture fluid in which 200 chilomonads had been for 4 hours (i.e. the minimum concentration which causes retardation in the rate of reproduction) would be approximately 1 part in 4,000,000.

While it is obvious that all these results are crude approximations, they nevertheless show that substance X is effective in extremely low concentrations in the culture fluid.

RELATION BETWEEN CONCENTRATION IN PROTOPLASM OF SUBSTANCE X PRODUCED BY *Chilomonas* AND RATE OF REPRODUCTION

In the preceding sections it was demonstrated that the rate of reproduction of *Chilomonas* is closely correlated with the concentration of X in the culture fluid, but the results show that there was great variation in the rate of reproduction in experiments made on different days with culture fluid in which the concentration of X was the same. This becomes obvious if the results presented in the preceding tables are compared. Tables 7, 9, and 10 for example, show that the time required for the first division after the chilomonads had been transferred to fresh acetate-ammonium solution in the different experiments made varied from 5.75 to 7.41 hours.

The chilomonads used in all the experiments were taken from vigorous cultures and were carefully selected for size. The temperature and the culture fluid were practically the same. What, then, was the cause of the variation in the rate of reproduction observed? The following experiments concern this question.

Experiment 1.—Four groups of 12 isolation cultures each were set up in depressions on slides. Each culture contained 0.025 cc acetate-ammonium culture fluid and 1 small chilomonad, selected for size and content from a young flourishing culture.[4] All the cultures were kept in the water-bath at 24.4 ± 0.1° C. and examined every hour until each chilomonad had divided twice. Then the average time required for the first and for the second division, respectively, was calculated for the 12 chilomonads in each group. It was found that the first division required respectively 6.20, 6.10, 6.00, and 6.25 hours in the four groups and the second division, respectively 5.25, 5.16, 5.45, and 5.25 hours.

These results show that if several groups of chilomonads are selected from a given culture at the same time, the average rate of reproduction in the different groups is fairly uniform; and they indicate that the excessive variation observed in the preceding experiments must in some way have been correlated with the fact that the chilomonads used were taken from different cultures, despite the fact that all these cultures were in a flourishing condition.

The results presented also show that, after the chilomonads have been transferred from an acetate-ammonium culture fluid in which they had grown for some time and which consequently contains considerable substance X, to fresh acetate-ammonium culture fluid which contains no X, the first division requires much more time than the second division.

---

[4] Before the chilomonads were put into the isolation cultures, they were passed successively thru each of 4 portions of sterile fresh culture fluid in depressions on slides.

This is what would be expected if the chilomonads contained, in their protoplasm, X in supraoptimum concentration when they were transferred from the old to the fresh culture fluid (as was probably true) and if, after they were transferred, some of X diffused out so as to decrease its concentration in the protoplasm and increase it in the culture fluid, thus making it more nearly optimum in both, and consequently causing an increase in the rate of multiplication.

Experiment 2.—Approximately 500 chilomonads were taken from a culture which was 3 days old and put into a depression on a slide which contained 0.1 cc acetate-ammonium culture fluid. This culture was then put into the water-bath at 24.5 ± 0.2° C. and left 24 hours; then 500 of the chilomonads from this culture were transferred to each of two depressions, each containing 0.1 cc culture fluid, and left 24 hours; after which 500 were transferred from one of these depressions to each of two others, each containing 0.1 cc culture fluid. This was repeated 24 hours later, resulting in three cultures which were precisely the same except that the first was 1 day older than the second, and the second 1 day older than the third. These three cultures were left in the water-bath 24 hours, then 12 small chilomonads which had recently divided were selected from those in each culture, washed as described above, and put respectively into 0.025 cc culture fluid in depressions on slides in petri dishes in the water-bath, after which they were examined every hour and the average time required for division in each group calculated.

It was found that those from the one-day-old culture required an average of 6.08 hours; those from the two-day-old culture, 7.75 hours; and those from the three-day-old culture, 7.91 hours.

This experiment was repeated several days later and the averages obtained were 6.25, 7.08, and 7.33 hours, respectively.

This shows that the rate of reproduction in chilomonads which have been transferred to fresh culture fluid is closely correlated with the age of the culture from which they are taken.

We have demonstrated that the concentration in the culture fluid of substance X varies directly with the age of the culture and the density of the population. It was consequently much more concentrated in the three-day-old than in the one-day-old culture, and the protoplasm in the former doubtless contained much more than that in the latter. The concentration in all three cultures was supraoptimum, for in the youngest culture it equaled the amount produced in 0.1 cc by 500 chilomonads in 24 hours or approximately 28,000 chilomonad-hours per 0.1 cc; whereas the optimum, as shown above, is approximately only 300 chilomonad-hours per 0.1 cc. In the oldest culture it was, however, very much higher, i.e. over 2,000,000 chilomonad-hours per 0.1 cc.

We would therefore expect the time required for division in fresh culture fluid to be much shorter in the former than in the latter, which is precisely what was observed.

The variation in the rate of reproduction in fresh culture fluid with the age of the culture from which the chilomonads have been taken appears therefore to be due to differences in the amount of X in the protoplasm carried over in the transfer. This conclusion is strongly supported by the results obtained in the following experiment.

Experiment 3.—Five hundred chilomonads were taken from a vigorous culture and put into 0.1 cc fresh acetate-ammonium culture fluid in a depression on a slide in the water-bath at 24.5 ± 0.2° C. Four days later 500 more were taken from a similar culture and treated in the same way and 1 day later one from another similar culture. One day after this, 12 small individuals which had recently divided were taken from each of these three depression cultures, washed as in the preceding experiments, and put separately into 0.025 cc culture fluid in each of 36 depressions on slides in petri dishes. This resulted in 12 isolation cultures, each of which contained 1 chilomonad from a six-day-old culture containing very many specimens, 12 which contained chilomonads from a two-day-old culture containing a moderate number, and 12 which contained chilomonads from a two-day-old culture containing only a few (about 64). The concentration of X in the protoplasm of the chilomonads in the first group of cultures was therefore "strong," that in the protoplasm of those in the second group "medium," and that in the protoplasm of those in the third group "weak." All these cultures were kept in the water-bath and examined hourly.

After the first division of the chilomonad in each of these cultures, one was removed and put into 0.025 cc fresh culture fluid in a clean depression on a slide in a petri dish, and the other was left in the old culture fluid. After all had divided, there were therefore 36 isolation cultures which contained one-day-old culture fluid and 36 which contained fresh culture fluid. The former were designated "A," and the latter "B." All were put into the water-bath and then examined hourly. In A, 1 chilomonad was removed and discarded after each division, and the culture fluid removed (only a small amount) with it was replaced with fresh culture fluid. In B, 1 chilomonad was removed after each division and put into 0.025 cc fresh culture fluid in a clean depression, and the other one discarded. The results obtained are presented in table 11 and figure 2.

This table shows that the time required for the first division varied greatly, i.e. from 6.08 to 10.10 hours, and that it varied inversely with the concentration of X in the protoplasm of the chilomonads.[5] It also shows that in both A and B, as the time increased, the rate of reproduction under all three conditions increased to a maximum and then decreased but that the rate of decrease was very much more rapid and the maximum rate considerably higher in the chilomonads in which the concentration of X was "strong" than in those in which it was "weak" and that the maximum rate of reproduction was, in general, probably reached a little sooner in A than in B. It shows however, that at the close of the experiment the rate was very nearly the same under the three conditions but that it was a little higher in A than in B. The table indicates that in the chilomonads in which the concentration of X was weakest there was a slight decrease in the rate of reproduction before the increase to a maximum occurred and that this decrease was considerably higher in B than in A. All these results become much more evident by referring to and comparing the curves in figure 2. What do they signify?

The concentration of substance X in the culture fluid from which the chilomonads used in this experiment were taken was approximately 504 chilomonad-hours per 0.1 cc in "weak," 252,000 in "medium," and more than 1,000,000,000 in "strong." It was therefore supraoptimum in all, for the optimum is only 300 (Table 10); and the concentration in the protoplasm of the chilomonads was probably somewhat higher than that in the culture fluid. In the transfer of the chilomonads from the old culture fluid to fresh culture fluid the X in the protoplasm was of course also transferred; one would therefore expect the rate of reproduction in the fresh culture fluid immediately after the chilomonads were transferred to it to vary inversely with the concentration of X in their protoplasm in accord with the results obtained. And since, after the transfer, X doubtless (owing to difference in concentration) diffused out of the protoplasm and became weaker and weaker, reaching optimum concentration and then suboptimum, one would expect that, as the time in fresh culture fluid increased, the rate of reproduction under all three conditions investigated would increase to a maximum and then decrease, which is precisely what was observed; and one would expect the maximum rate to be reached a little later and the rate at the close of the experiment to be a little higher in A than in B, for the concentration of X was, after the first division, continuously a little higher in A than in B, owing to the fact that culture fluid was not changed in A. The latter expectation is in accord with what was observed but the former probably is not, although there is some doubt concerning this.

---

[5] This experiment was in part repeated twice. In one test the average time required for the first division was 7 hours with chilomonads from a young culture and 10.20 with chilomonads from an old culture, and in the other it was 6.41 and 10.80 hours, respectively.

TABLE 11

THE RELATION BETWEEN THE CONCENTRATION OF SUBSTANCE X IN THE PROTOPLASM OF *Chilomonas paramecium* AND THE RATE OF REPRODUCTION IN FRESH ACETATE-AMMONIUM CULTURE FLUID

Concentration of X in protoplasm: weak, chilomonads taken from a culture started with 1 chilomonad in 0.1 cc acetate-ammonium culture fluid and left 2 days; medium, chilomonads taken from a culture started with 500 in 0.1 cc acetate-ammonium fluid and left 2 days; strong, chilomonads taken from a culture started with 500 in 0.1 cc and left 6 days. Temperature, 24.5 ± 0.2° C.; hydrogen-ion concentration, pH 6.8; Chi., chilomonads.

In experiment A, after each division, one of the chilomonads was removed from the culture and discarded. In experiment B, after each division, one of the chilomonads was put into a clean depression containing 0.025 cc fresh acetate-ammonium culture fluid and the other one discarded.

Concentration of Substance X in Protoplasm of Chi.	Average Time (in hours) for 12 Chi. in Isolation Cultures							
	To First Division	First to Second Division	Second to Third Division	Third to Fourth Division	Fourth to Fifth Division	Fifth to Sixth Division	Sixth to Seventh Division	Seventh to Eighth Division
	A							
Weak	6.08	6.00	6.33	5.08	4.83	5.08	5.66	5.90
Medium	7.60	5.66	5.58	5.00	5.00	4.45	4.66	6.00?
Strong	10.10	5.90	4.33	4.87	4.37	4.28	5.00	5.80?
	B							
Weak	6.08	6.25	6.75	5.83	5.50	5.41	5.83	....
Medium	7.60	4.70	5.50	5.90	5.20	5.30	6.66	6.40?
Strong	10.10	5.90	5.00	5.22	4.50	5.00	5.50	6.50?

The correspondence between expected and observed strongly supports the conclusions reached from the results obtained in the preceding experiment, namely, that the rate of reproduction in *Chilomonas* is closely correlated with the concentration of X in the protoplasm and that the variation in the rate of reproduction in fresh culture fluid observed in the experiments described in the preceding sections of this paper was due to variation in the concentration of X in the culture fluid from which the chilomonads used were taken.

But why the rate of reproduction in the experiment now under consideration increased much more rapidly in chilomonads transferred from old than in those transferred from young culture fluid to fresh culture fluid and why the maximum rate was higher in the former than in the latter and why there was at first decrease in the latter and none in the former and why this decrease was greater in B than in A is not clear. There are, of course, various other factors besides X involved in the control of the rate of reproduction in *Chilomonas*, and their action doubtless accounts for these results.

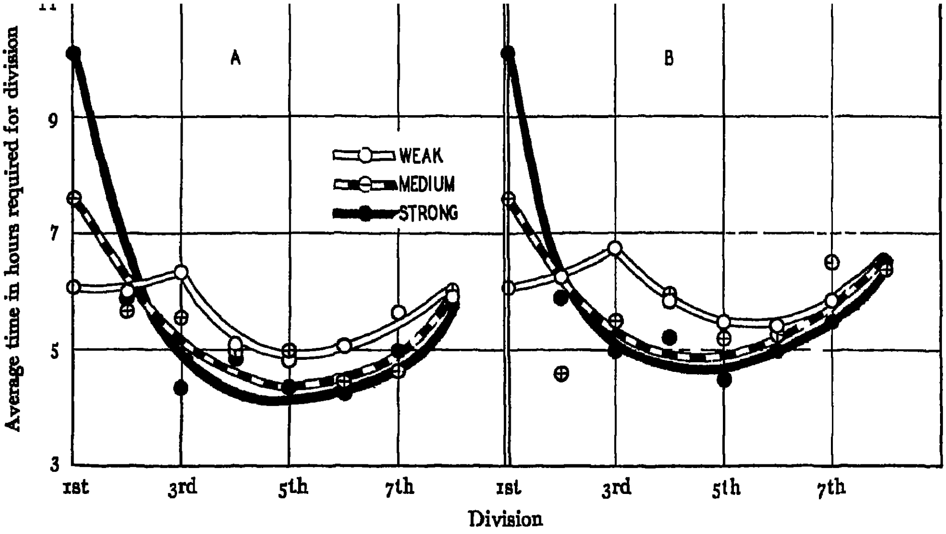


FIG. 2.—The relation between the concentration of substance X in the protoplasm of *Chilomonas paramecium* and the rate of reproduction in fresh acetate-ammonium culture fluid. Each point on the curves represents the average time required for division of 12 individuals. ○, chilomonads from a young culture; ⊖, chilomonads from a medium-aged culture; ●, chilomonads from an old culture. In experiment A, after each division, one of the chilomonads was removed from the culture and discarded. In experiment B, after each division, one of the chilomonads was put into a clean depression containing 0.025 cc fresh acetate-ammonium solution.

Experiment 4.—If X readily diffuses out of the chilomonads, and if the rate of reproduction is closely correlated with the concentration of X in the protoplasm in accord with the postulations made above, one would expect the rate of reproduction to vary inversely with the frequency of transfer to fresh culture fluid.

Two series of observations were made concerning this hypothesis. In one, 24 chilomonads were selected for size and content from a vigorous culture and washed in fresh culture fluid, as described above. One of these was put into each of 24 depressions containing 0.025 cc culture fluid each and transferred to the water-bath. Every half-hour after this the chilomonads in 12 of the depressions were transferred to fresh culture fluid in clean depressions and those in the other 12 depressions were taken up in a pipet and returned to the solution from which they had been taken. The average time required for the first division was 11 hours for those which had been transferred to fresh culture fluid and only 6.6 for those which had not.

In the second series of observations the methods used were the same as those in the first except that 12 of the chilomonads used were transferred to fresh culture fluid every 15 minutes in place of every 30 minutes. These observations were continued for 15 hours and during that time none of those which had been transferred to fresh culture fluid divided, whereas the average time required for the first division in the twelve which had not been transferred was only 6.25 hours.

The results obtained indicate therefore that the rate of reproduction in *Chilomonas* varies inversely with the frequency of transfer to fresh culture fluid, and they consequently support the conclusion concerning the effect of diffusion of X on the rate of reproduction, reached in the preceding experiments.

DISCUSSION

As previously stated, Robertson concluded that unicellular organisms and probably all other cells produce substances which accelerate growth; Yocom, Petersen, and Dimitrowa obtained results which support this conclusion; and Woodruff, Peskett, Greenleaf, Calkins, Myers, Grimwald, Chejfec, Jahn, Darby, Di Tomo, Beers, Johnson and Phelps obtained results which oppose it, and they conclude that protozoa do not produce growth-promoting substance and imply that the reverse results obtained by Robertson and others are due to differences in the amount or the kind or the availability of the food under the different conditions which obtained in the experiments.

The results presented above demonstrate that the rate of reproduction in *C. paramecium* varies directly with the density of population under some conditions and inversely under others, and that these results are not dependent upon variation in the amount, the kind, or the availability of food but upon the concentration of an unknown substance (X) produced by the chilomonads. However, the fact that such substance is produced by *Chilomonas* obviously does not prove that similar substance is produced by other cells and it therefore does not prove that Robertson's conclusion is valid; but it nevertheless does lend some support to this conclusion. And the facts that Went (1937) and others, as is well known, demonstrated that growth-promoting substance is produced in some plant cells, and that Peebles (1929) and others obtained results which appear to indicate that such substance is produced by eggs, also lend support to it. Moreover, we demonstrated that the rate of reproduction in fresh culture fluid varies with the age of the parent culture. The divergent results obtained by others on the relation between density of population and rate of reproduction may consequently be due—at least in part—to difference in the age of the parent cultures rather than to difference in the food. These results therefore do not necessarily overthrow Robertson's conclusion.

The results presented in the preceding section of this paper seem to prove that *Chilomonas* not only produces growth-promoting substance in accord with Robertson's views but that if this substance is concentrated, it retards and inhibits growth and kills the organisms. If the production of such a substance is widespread among cells, it is obviously of the greatest importance in all problems concerning growth, reproduction, and senescence, and it doubtless has an important bearing on many others.

We are continuing our experiments on *Chilomonas* concerning the nature and the action of the substance X produced by them, and we hope soon to extend them to other forms.

SUMMARY

1. Sterile acetate-ammonium culture fluid and the isolation culture method, with transfer to fresh culture fluid every 24 hours, were used practically exclusively in this work.

2. *Chilomonas* grows indefinitely in mass cultures in acetate-ammonium culture fluid if it is transferred to fresh fluid every other day; but if it is not transferred, it dies out in about 6 days.

3. In isolation cultures transferred to fresh culture fluid every 24 hours the rate of reproduction of *Chilomonas* increases to a maximum as the volume of culture fluid per individual decreases and then decreases to zero.

4. The average number of chilomonads produced in 24 hours at 24 ± 0.2° C., from each specimen introduced, increased in one series of tests from 8.7 with 1 specimen in 0.4 cc culture fluid to 13.65 with 50 specimens in 0.4 cc (more than 50 percent) and in another from 9.15 with 1 specimen in 0.4 cc to 12.98 with 1 specimen in 0.006 cc (over 40 percent).

5. The increase in rate of growth is somewhat greater than the increase in rate of reproduction.

6. If different numbers of chilomonads are left for the same length of time in the same amount of culture fluid, and then removed and chilomonads from a vigorous culture added, the rate of reproduction in the culture fluid increases to a maximum, as the number of chilomonads which had been in it increases, and then decreases to zero.

7. If culture fluid in which *Chilomonas* has died out is subjected to 100° and then cooled, the rate of reproduction of *Chilomonas* in it increases from zero to maximum, as the time at 100° increases, and then decreases to that which obtains in fresh culture fluid.

8. If culture fluid in which *Chilomonas* has died out is diluted with fresh culture fluid, the rate of reproduction of *Chilomonas* in it increases to maximum, as the dilution increases, and then decreases to normal.

9. The relation between the number of chilomonads per given volume of culture fluid and the rate of reproduction is not due to change in the culture fluid, owing to consumption of substance in it or to mitogenetic rays or to contact stimulation. It is correlated with the concentration of a substance produced by the chilomonads.

10. This substance (designated "X") accelerates reproduction in low concentrations and inhibits it and causes death in high concentrations.

11. Fission is not necessary for the production of substance X.

12. The lowest effective concentration of X is produced by one chilomonad in 0.1 cc culture fluid for 4 hours, and the optimum concentration by 75 in 0.1 cc for 4 hours.

13. If 1 chilomonad produces X equal in volume to one-tenth of its volume in 4 hours, the lowest effective concentration is about 1 part X in 800,000,000 parts of culture fluid and the optimum is about 1 part in 10,000,000.

14. If culture fluid in which chilomonads have developed long enough to produce a maximum rate of reproduction (i.e. X in optimum concentration) is subjected to 100° C. for 60 minutes, the rate in it is the same as in fresh culture fluid. If culture fluid in which chilomonads have died out is subjected to 100° C. for 90 minutes, the rate in it is the same as it is in fresh culture fluid.

15. The substance X produced by *Chilomonas* is heat-labile, and the time required to destroy it in a given temperature varies directly with the concentration.

16. The rate of reproduction of chilomonads in culture fluid containing X in a given concentration, varies with the age of the culture from which they were taken. This is due to variation in the concentration of X in the protoplasm, with the age of the culture.

17. If chilomonads in which the concentration of X in the protoplasm is supra-optimum are transferred to fresh culture fluid and then transferred every 24 hours, the rate of reproduction increases to a maximum and then decreases to normal. This is due to decrease in the concentration of X in the protoplasm, owing to diffusion.

18. If chilomonads are frequently changed to fresh culture fluid, the rate of reproduction decreases as the frequency increases. This is due to diffusion and consequent decreases of X in the protoplasm.

LITERATURE CITED

Beers, C D. 1933. The relation of density of population to rate of reproduction in the ciliates, *Didinium nasutum* and *Stylonychia pustulata*. Arch. f. Protistenk., 80:36–64.

Calkins, G. N. 1926. Biology of the protozoa. Philadelphia and New York. Pp. 623.

Chejfec, M. 1929. Die Lebensdauer von *Paramecium caudatum* in Abhangigkeit von der Nahrungsmenge. Acta biol. exper., 4:73–118.

Cutler, D. W., and Crump, L. M. 1923, 1924. The rate of reproduction in artificial cultures of *Colpidium colpoda*. Biochem. Jour., 17:174–86, 878–86; 18:905–12.

———. 1925. The influence of washing upon the reproductive rate of *Colpidium colpoda*. *Ibid.*, 19: 450–53.

Darby, H. H. 1930. Studies on growth acceleration in protozoa and yeast. Jour. Exper. Biol., 7:308–15.

Dimitrowa, A. 1932. Die fördernde Wirkung der Exkrete von *Paramecium caudatum* Ehrbg. auf dessen Teilungsgeschwindigkeit. Zool. Anz., 100:127–32.

Greenleaf, W. E. 1924. The influence of volume of culture medium and cell proximity on the rate of reproduction of protozoa. Proc. Soc. Exper. Biol. and Med., 21:405–6.

———. 1926. The influence of volume of culture medium and cell proximity on the rate of reproduction of infusoria. Jour. Exper. Zoöl., 46:143–67.

Grimwald, E. 1928. Recherches sur les facteurs du développement des cultures de micro-organismes. L'Action de la substance allelocatalytique apparaît-elle dans les cultures du *Colpidium colpoda* Ehrb. Acta biol. exper., 3:81–100.

Jahn, T. L. 1929. Studies on the physiology of the euglenoid flagellates. I. The relation of the density of population to the growth rate of *Euglena*. Biol. Bull., 57:81–106.

Johnson, W. H. 1933. Effect of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., 6:22–54.

Mast, S. O. 1928. Factors involved in changes in form of *Amoeba*. Jour. Exper. Zoöl., 51:97–120.

Mast, S. O., and Pace, D. M. 1933. Synthesis from inorganic compounds of starch, fats, proteins and protoplasm in the colorless animal, *Chilomonas paramecium*. Protoplasma, 20:326–58.

———. 1935. Relation between sulfur in various chemical forms and the rate of growth in the colorless flagellate, *Chilomonas paramecium*. *Ibid.*, 23:297–325.

———. 1937. The effect of silicon on growth and respiration in *Chilomonas paramecium*. Jour. Cell. and Comp. Physiol., 10:1–13.

Mast, S. O.; Pace, D. M.; and Mast, L. R. 1936. The effect of sulfur on the rate of respiration and on the respiratory quotient in *Chilomonas paramecium*. Jour. Cell. and Comp. Physiol., 8:125–39.

Myers, E. C. 1927. Relation of density of population and certain other factors to survival and reproduction in different biotypes of *Paramecium caudatum*. Jour. Exper. Zoöl., 49:1–43.

Peebles, F. 1929. Growth regulating substances in echinoderm larvae. Biol. Bull., 52:176–87.

Peskett, G. L. 1924. Allelocatalysis and the growth of yeast. Jour. Physiol., 59:33.

———. 1924. Allelocatalysis and the growth of yeast. Biochem. Jour., 18:866–71.

———. 1925. Studies on the growth of yeast. I. The influence of the volume of culture medium employed. *Ibid.*, 19:464–73.

1925. Studies on the growth of yeast. II. A further note on allelocatalysis. *Ibid.*, pp. 474–76.

Petersen, W. A. 1929. The relation of density of population to rate of reproduction in *Paramecium caudatum*. Physiol. Zoöl., 2:221–54.

Phelps, A. 1935. Growth of protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and of the amount of the inoculum. Jour. Exper. Zool., 70:109–30.

———. 1936. Growth of protozoa in pure culture. II. Effect upon the growth curve of different concentrations of nutrient materials. Jour. Exper. Zool., 72:479–96.

Robertson, T. B. 1921. Experimental studies on cellular multiplication. I. The multiplication of isolated infusoria. Biochem. Jour., 15:595–611.

———. 1921. Experimental studies on cellular multiplication. II. The influence of mutual contiguity upon reproductive rate and the part played therein by the "X-substance" in bacterial infusions which stimulates the multiplication of Infusoria. *Ibid.*, pp. 612–19.

———. 1922. Reproduction in cell communities. Jour. Physiol., 56:404–12.

———. 1923. The chemical basis of growth and senescence. Philadelphia and London. Pp. 389.

———. 1924. Allelocatalytic effect in cultures of *Colpidium* in hay infusions and in synthetic medium. Biochem. Jour., 18:1240–47.

Tomo, M. di. 1932. Ricerche sul comportamento di *Paramecium caudatum* in un dato volume di cultura liquida. Boll. di zool., 3:137–40.

Went, F. W., and Thimann, K. V. 1937. Phytohormones. New York. Pp. 204.

Woodruff, L. L. 1911. The effect of excretion products of *Paramecium* on its rate of reproduction. Jour. Exper. Zool., 10:557–81.

———. 1913. The effect of excretion products of infusoria on the same and on different species with special reference to the protozoan sequence in infusions. *Ibid.*, 14:575–82.

Yocom, H. B. 1928. The effect of the quantity of culture medium on the division rate of *Oxytricha*. Biol. Bull., 54:410–17.

FAT RELEASE IN AMOEBA AFTER IRRADIATION[1]

(One plate)

L. V. HEILBRUNN AND KATHRYN DAUGHERTY
University of Pennsylvania

IT HAS been shown that ultra-violet radiation and also other stimulating agents cause a release of calcium in protoplasm (Heilbrunn and Daugherty, 1933; Heilbrunn and Mazia, 1936; Mazia and Clark, 1936). This is doubtless not the only effect of such agents. Nadson (1925) found that radium caused a production of fat droplets in the protoplasm of yeast cells and that the same effect was also produced by ultra-violet radiation. In general, it appears that various types of irradiation may release fat or lipoid from protoplasmic combination (for references to literature see Heilbrunn and Mazia, 1936). The evidence on which this statement is based is perhaps not too clear.

By centrifuging cells, it is possible to estimate the amount of free fat in them (Heilbrunn, 1936). In this study we have attempted to discover whether radiation from a mercury arc lamp can cause an increase in the amount of fat which can be centrifuged to one side of a cell.

The work was done on two species of amoeba, *Amoeba proteus* and *A. dubia*. The results were essentially the same for both species, but only data for *A. proteus* are presented. The organisms were exposed for varying lengths of time to the complete radiation from a Uviarc lamp kindly loaned by the General Electric Company. The lamp was run at 100 volts and 3.8 amperes, and the amoebae were placed at a distance of 40–45 cm. from the arc. No attempt was made to determine the intensity of the radiation employed or to isolate particular wave-lengths. The determination of radiation intensity is not simple. Isolation of individual wave-lengths so weakens the intensity that it is difficult to obtain effects on biological material. In our experiments we were not concerned with the relative effect of one wave-length as compared with another, nor were we interested in discovering the intensity necessary to produce an effect. The Uviarc lamp is well known, and its spectrum has been carefully studied (Buttolph, 1930).

In our experiments we sought to use an effective but not lethal intensity of radiation, and we wished to discover whether treatment with such radiation would actually cause a release of fat in amoeba. In experiments of this sort, it is possible to make a rough quantitative measure of the free fat in individual amoebae and to compare the amount in normal and irradiated specimens. If amoebae which normally contain a small amount of free fat are exposed to the radiation from a Uviarc lamp, it can be shown that the amount of the free fat increases following irradiation. However, these experiments are not very satisfactory, for it is difficult to obtain anything better than a rough estimate of the volume of free fat present in an amoeba. It is simpler and more conclusive to conduct experiments on strains or cultures of amoebae which normally are totally lacking in free fat. Such fat-free cultures must be carefully selected, for they are the exception rather

---

[1] This work has been aided by a grant from the Radiation Committee of the National Research Council.

than the rule. Although we always began with fat-free cultures of amoebae, in some of our experiments, we found it worth while to make conditions favorable to fat release before exposing the amoebae to the Uviarc lamp. This was done by immersing the amoebae for hours or even days in distilled water. Of the amoebae in distilled water, a small percentage showed free fat. This percentage was greatly increased after the irradiation.

TABLE 1

FAT RELEASE AFTER 45 SECONDS OF EXPOSURE TO ULTRA-VIOLET IRRADIATION

Date (1936)	Temp. (°C.)	pH of Culture Medium	Culture No.	Control Amoebae without Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	Percentage of Amoebae Showing Fat Release
June 15......	24	7.3	(1)	35−	15–30	11−, 2+	
		7.3	(5)	27−	15–30	5−, 4+	
		....	(1)	....	45–60	..... 5+	
		....	(5)	....	45–60	..... 11+	
16......	24.5	....	(1)	4−	60–75	3−, 4+	
		....	(5)	5−	60–75	1−, 3+	
19......	26.0	....	(10)	4−	60	5−	
22......	24.0	....	(8)	4−	45–75	16−, 3+	
24......	22.5	....	(8)	8−	60–75	6−, 5+	
			(10)	11−	75	5−	
25......	22.5	....	(8)	8−	45–60	12−, 3+	
Total....	....	....	....	106−	....	104 (40+)	38.4
Nov. 11......	18.5	....	I	9−	60	4−, 4+	
		7.2	(4)	7−	60	8−	
		7.4	(2)	10−	60	7−	
12......	20.0	....	(2)	28−	60	11−	
30......	20.0	6.9	(2)	4−	60	1−, 2+	
		....	....	....	26	10−, 3+	
Dec. 2......	20.0	6.9	III	65−	30–45	35−	
		....	....	....	70	12−	
		....	....	....	90–120	35−	
Total....	....	....	....	123−(0%)	....	132 (9+)	6.8

The technique of the experiments was very simple. After exposure to the Uviarc lamp, the amoebae were allowed to remain in suitable dishes for 30–60 minutes. Such a time interval is necessary to allow the process of fat release to take place. At the expiration of the time interval, the amoebae, both irradiated specimens and controls, were centrifuged for 5–10 minutes in an Emerson electric centrifuge at a force approximately six thousand times gravity. The centrifugal treatment was strong enough to stratify the protoplasmic constituents, and the fats of the cell were thrown to the light pole. Here they could readily be stained with Sudan III. This is illustrated in Plate I. In the upper portion of the irradiated cells, there is an accumulation of dark material, which is colored a brilliant orange in the original preparation. No such material appears in the control.

The tables show three series of experiments, and they give the results of exposures of 45, 60, and 90 seconds. In all the experiments irradiation did cause a very definite release of fat in a fair percentage of exposed individuals. This percentage was always greater when the experiments were conducted in the warmer months. Thus, in Table 1 the June amoebae showed 38.4 per cent with positive results, whereas the November and December amoebae treated in the same way showed only 6.8 per cent.

TABLE 2

FAT RELEASE AFTER 60 SECONDS OF EXPOSURE TO ULTRA-VIOLET IRRADIATION

Date (1936)	Temp. (°C.)	pH of Culture Medium	Culture No.	Control Amoebae without Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	pH of Distilled Water	Time in Distilled Water	Control Amoebae with (+) or without (−) Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	Percentage with Fat Release
Nov. 12...	20	...	(2)	28−	60	25− ...	....	......	... ...	....	....	
		.....	(4)	34−	60	21− ...	....	......	... ...	....	....	
16...	19.5	7 4	(2)	15−	....	....	6 8	68 hr.	6− ...	76	4−, 2+	
17...	21.5	7.2	(2)	9−	....	....	....	2 hr.	7− ...	50	2−, 4+	
21...	20.0	7.5	(4)	15−	....	....	7 0	21 hr.	7−, 4+	60	0−, 2+	
23...	21.5	7.0	(2)	15−	....	....	6.5	48 hr.	13− ...	50	.... 4+	
24...	20–22	7.2	(1)	10−	62	13−, ...	....	....	....	....	....	
		7.3	(2)	12−	60	4−, 1+	....	....	....	....	....	
30...	20	6.9	(2)	4−	90	11−, 2+	....	....	....	....	....	
Dec. 2...	20	6.9	III	65−	70	18− ...	....	....	....	....	....	
3...	21	6.7	III	11−	....	....	6 5	5½ hr.	18− ...	44	.... 7+	
4...	23	6.9	III	25−	58	21− ...	6.5	22 hr.	9− ...	60–65	.... 35+	
		.....	.....	....	....	....	....	......	....	30–45	.... 19+	
		.....	.....	....	... .	....	....	4 hr.	20− ...	60	23−, 2+	
7...	20	7 2	(4)	13−	37	5−, 3+	....	....	....	....	....	
		7 0	III	20−	37	7− ...	6 5	3 days	12− ...	42	5−, 3+	
8...	19–21	7.0	III	....	....	....	6.3	24 hr.	6− ...	39	6−, 1+	
		.....	.....	....	....	....	....	....	....	80	1−, 1+	
		6.9	(2)	....	125	5−, 2+	....	....	....	....	....	
		.....	(4)	6−	60	16− ...	6 3	24 hr.	7− ...	125	3−, 2+	
		.....	.....	....	130	7−, 5+	....	... ..	....	....	....	
Total	......	.....	.....	282 (0%+)	....	166 13+= (7.8%+)	....	....	109 4+= (3.6%+)	....	132 82+=	62.1

Tables 2 and 3 give data both for amoebae irradiated directly in culture fluid and for amoebae immersed in distilled water before exposure to the radiation. Following exposure to distilled water, much higher percentages of amoebae with free fat are obtained. The favorable effect of the distilled water is perhaps to be correlated with the fact that such water is lacking in carbonates and bicarbonates. On the other hand, the culture fluid, which consisted essentially of spring water, presumably did contain carbonate or bicarbonate. The presence of such compounds would tend to send carbonic acid into the protoplasm (Jacobs, 1920). Thus, the amoebae in distilled water might have less acid protoplasm than those in culture fluid. It has been shown previously (for the protoplasm of sea-urchin eggs) that acidity tends to prevent fat release (Heilbrunn, 1936). Moreover, in the case of amoeba, we were able to show that treatment with carbon dioxide prevented release of fat by irradiation. On the other hand, if amoeba protoplasm is made more alkaline by immersion in dilute ammonium chloride solutions, then subsequent irradiation causes a greatly increased release of fat and the entire amoeba becomes filled with tiny droplets, presumably of fatty material.

As a whole, the experiments confirm the older observations which have shown that fat release may follow irradiation of protoplasm. Further study is necessary in order to determine whether this fat release is due to a direct action of the radiation on the protein-lipid binding or whether it may not be due indirectly to an alkalinization of the protoplasm. For it is conceivable that irradiation makes the protoplasm more alkaline and that this alkalinization produces a release of bound fat.

TABLE 3

FAT RELEASE AFTER 90 SECONDS OF EXPOSURE TO ULTRA-VIOLET IRRADIATION

Date (1936)	Temp. (°C.)	pH of Culture Medium	Culture No.	Control Amoebae without Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	pH of Distilled Water	Time in Distilled Water	Control Amoebae with (+) or without (−) Fat Release	Time between Exposure and Centrifuging (Min.)	Amoebae with (+) or without (−) Fat Release	Percentage with Fat Release
Nov. 13 ..	19	7.4	(2)	....	90	6−, 1+	....	....	....	....	....	
		7.6	(4)	5−	95	2− ...	....	....	....	....	....	
17...	21 5	7.2	(2)	9−	93	4−, 1+	....	....	....	....	....	
18...	21.0	7.7	(4)	4−	60	.... 3+	7.0	22 hr.	2−, 3+	30	.... 5+	
21 ..	20 0	7.2	(2)	20−	....	....	7.0	21 hr.	16−, ...	75	.... 9+	
		7.5	(4)	15−	60	10− ...	7.0	21 hr.	7−, 4+	75	1−, 2+	
23...	21.5	7.0	(2)	15−	30–60	23− ...	6.5	48 hr.	13− ...	30–60	10−	
24..	20–22	7.0	(2)	12−	70	19− ...	6.5	19 hr.	13− ...	60	.... 10+	
		7.3	(4)	12−	70	7−, 2+	....	....	....	....	....	
30...	20.0	6.9	(2)	4−	75	8− ...	6.5	5 days	9− ...	28	11−, 3+	
		7.2	(4)	5−	75	12−, 3+	....	....	....	....	....	
Dec. 2...	20 0	6 9	III	65−	70	10− ...	....	....	....	....	....	
3 .	21.0	6.9	(4)	13−	120	6− ...	....	....	....	....	....	
4...	19–21	7.1	(4)	6−	90	5−, 1+	....	....	....	....	....	
10...	21 0	....	III	12−	....	....	....	43 hr.	8−, 1+	68	3−, 5+	
Total.	......	.....	.....	197 (0%+)	....	123 11+ = (8.9%+)	....	....	76 8+ = (10%+)	....	59 34+ =	57.6

Irradiation is one type of stimulation. It seems not unlikely that other types of stimulation may also cause a release of fat in the protoplasm. This question offers possibilities for further research, and such work is at present being attempted in this laboratory. Preliminary results indicate that protoplasmic protein may be combined with both fat and calcium, and that both fat and calcium may be released in stimulation. Those types of stimulation which may involve localized increases in temperature seem especially prone to release fat.

SUMMARY

Radiation from a mercury arc lamp causes a release of fat in amoeba protoplasm.

LITERATURE CITED

BUTTOLPH, L. I. 1930. The electrical characteristics of commercial mercury arcs. Rev. Sci. Instruments, 1:487.

HEILBRUNN, L. V. 1936. Protein lipid binding in protoplasm. Biol. Bull., 73:557.

HEILBRUNN, L. V., and DAUGHERTY, KATHRYN. 1933. The action of ultraviolet rays on amoeba protoplasm. Protoplasma, 18:596.

HEILBRUNN, L. V., and MAZIA, D. 1936. The action of radiations on living protoplasm. In Duggar, Biological effects of radiation, 1:625. New York, 1936.

JACOBS, M. H. 1920. The production of intracellular acidity by neutral and alkaline solutions containing carbon dioxide. Amer. Jour. Physiol., 53:457.

MAZIA, D., and CLARK, J. M. 1936. Free calcium and the action of stimulating agents on *Elodea* cells. Biol. Bull., 71:306.

NADSON, G. A. 1925. Über die Primärwirkung der Radiumstrahlen auf die lebendige Substanz. Biochem. Zeitschr., 155:381.

PLATE I

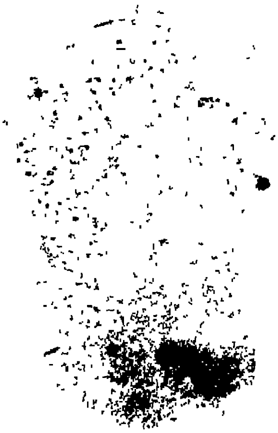
FIG. 1.—*Amoeba proteus*, centrifuged control. ×300.

FIG. 2.—*Amoeba proteus*, centrifuged after 45 seconds of irradiation. Time interval between irradiation and centrifugal treatment, 60 minutes. ×385.

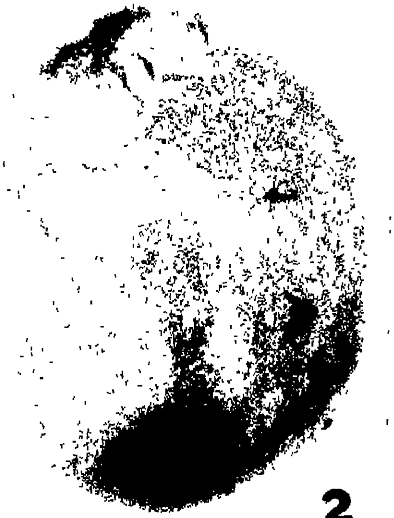
FIG. 3.—*Amoeba dubia*, centrifuged control. ×290.

FIG. 4.—*Amoeba dubia*, centrifuged after 45 seconds of irradiation. Time interval between irradiation and centrifugal treatment, 75 minutes. ×280.

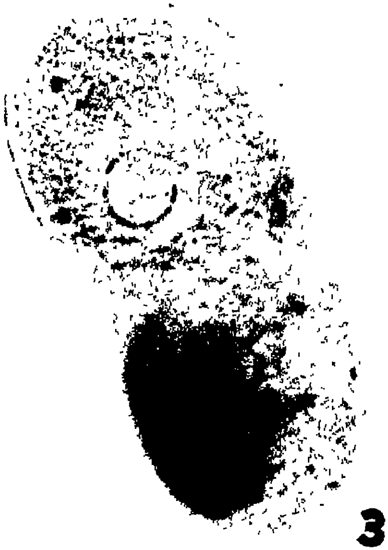
PLATE I



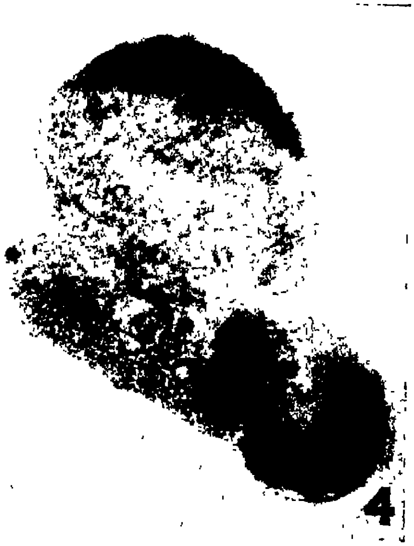
1



2



3



4

AN EXPERIMENTAL STUDY OF CLOT FORMATION IN THE PERIVISCERAL FLUID OF ARBACIA[1]

(One plate)

JAMES A. DONNELLON
Zoological Laboratory, University of Pennsylvania, and Marine Biological Laboratory,
Woods Hole, Massachusetts

IT IS well known that, if the body fluid of a sea urchin is withdrawn from the shell, it undergoes a change in which the cells separate completely from the fluid to form a clot. When the membrane or shell is injured in any way, the body fluid will clot at the point of injury and close in the opening (Kindred, 1921). Similar injury to the intestinal wall by sand or sharply pointed intestinal contents will also result in clot formation.

These observations suggested an investigation to determine, if possible, just why the body fluid should clot under these circumstances and not under normal conditions within the shell. In attempting to solve this problem, it was thought advisable to test the effect on the body fluid of different reagents, such as tissue extracts, fat solvents, salt solutions, or other reagents that might be suspected of being able to influence clot formation either by preventing or by causing it.

Previous investigators in this field have dealt more with the morphology of the clot than with the physiological problem suggested by its formation. Cuénot (1891) distinguishes two different series of cells from which he believes all the cellular elements are derived. The first series, which he calls "vibratile globules," are minute spherical elements with a head and vibratile tail. These cells rotate very rapidly and continuously, and their function, according to Cuénot, is to keep the fluid contents of the body cavity in motion.

The second series were called "amibocytes" and were subdivided into several different types. The cells of the first type of this series are the colorless "amibocytes" having a homogeneous cytoplasm and numerous filiform pseudopodia which sometimes unite to form complete rings. The name "leucocyte" was applied to these cells by Goodrich (1919), and many investigators believe that they are the chief agents in the formation of the clot. Further details concerning the morphology of clotting may be found in papers by Kindred (1921) and Theel (1896).

Geddes (1880) refers the clot formation to the remarkable massing-together of the leucocytes to form a network in which the other corpuscles become entangled. Kindred also observed this phenomenon but failed to determine whether these massed cells formed true plasmodia, as Geddes contends, or retained their individuality as later writers hold. E. A. Schäfer (1883) maintains that clotting is due to the exuding of what he calls "coagulable material" by the leucocytes when the body fluid is removed from the shell. This "coagulable material" is unlike the fibrin of vertebrate blood but appears to be closely allied to mucin. Schäfer's paper, however, was only a preliminary notice, and it seems that he never completed his investigation.[2]

---

[1] A thesis in zoölogy presented to the faculty of the graduate school of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

METHODS OF STUDY

The methods used in these experiments are comparatively simple. The procedure followed was to determine the difference in time of clotting of normal body fluid and of body fluid after treatment with different reagents. Equal amounts (8 cc.) of the body fluid from the same animal were placed in two different paraffined Stender dishes. Two cc. of the tissue extract or of the reagent to be tested were then added to the fluid in one container and an equal amount of sea water added, as a control, to the fluid in the second container. The difference in time of clotting was then observed. Clotting was determined to have taken place when the corpuscles were precipitated in typical clot formation. This precipitation is made more distinct by the accompanying pigment liberation. The end point is reasonably sharp and can scarcely be mistaken. Whenever necessary, clotting could be hastened by rotating the container. It was found to be practically impossible to remove the body fluid without its showing some signs of clotting. In order to prevent clotting from taking place too rapidly, it was found necessary to withdraw the fluid from the animal by means of a finely pointed, well-paraffined pipette. The point of this pipette had been rounded by heating in order to decrease the amount of injury to the membrane when the pipette was injected through it.

Another method was to inject 1 or 2 cc. of the reagent into the body cavity after first making a small opening in the membrane opposite to the point of injection in order to allow the escape of excess fluid. The membrane was then carefully removed to determine if the injected material had caused clotting within the shell.

RESULTS

Cellular nature of clot.—As already pointed out, certain observers had noted the cellular nature of the clot. It was thought possible, however, that in addition there might be some intercellular clotting. To determine if any such clotting took place, it was necessary to separate the cells from the fluid. This was accomplished by filtering the body fluid as it flowed from the shell. All the cells remained on the filter, and a clear fluid passed through. This fluid gave no coagulation on dilution; but, if the cells were suspended in water, they did form a typical clot. There was no plasma clot formation as is characteristic of certain other invertebrate forms.

Action of tissue extracts.—The well-known influence of tissue extract on vertebrate blood suggested testing the effect of extracts of invertebrate tissues on the body fluid of the sea urchin. These extracts were prepared by thoroughly grinding the tissues in the mortar crucible and then filtering with a suction filter.

A tissue extract of the shell of *Arbacia* minus the spines and all the internal structures causes clot formation within a few seconds. Extracts of the intestine or of any of the other internal structures will produce the same effect. These extracts are effective in dilutions of one to fifty.

Extracts of starfish, crustaceans, and mollusks gave the same results as *Arbacia* extracts. The crustacean extracts were muscle extracts, whereas extracts of the entire animal were used in other cases. Some of these extracts such as those of *Limulus*, the blue crab, *Venus mercenaria*, and *Mactra* were even more effective than *Arbacia* extracts. Extracts of frog's muscle had no effect on clot formation. Thrombin and cephalin likewise had no effect. The fact that vertebrate muscle extracts have no effect on invertebrate clotting has been reported by Nolf (1909).

---

[2] This problem was suggested by Dr. L. V. Heilbrunn. I wish to take this opportunity to express my appreciation for his kind advice and criticism.

All these extracts also brought about a liberation of the pigment of the cells, changing the color of the body fluid from red to orange.

An extract of previously clotted material seems to hasten clotting with the same effectiveness as extracts of *Arbacia* and other invertebrates. This extract was prepared by grinding a large amount of clotted material in a mortar crucible in which ground glass had been placed.

TABLE 1

Extracts	Average Time of Clotting (Seconds)	Control (Seconds)
Arbacia (shell)	52	3,318
Starfish	70	5,200
Limulus	28	4,098
Blue crab	34	3,024
Spider crab	49	3,150
Venus mercenaria	40	3,780
Mactra	38	2,760
Ostrea	58	4,356
Shrimp	83	5,130
Boiled *Arbacia* eggs	58	2,988
Broken *Arbacia* eggs	68	3,360
Clotted material	42	3,940
Sperm	96	4,632

Arbacia eggs which have been broken up in ground glass, or eggs which have been boiled, will also cause clot formation. Sperm extract has the same effect. Whole eggs which have not been boiled seem to have no effect on clot formation.

At least twenty-five experiments were performed with each extract. Concentrated extracts were used in all cases. The average time of clotting in these experiments is listed in Table 1.

The rather high average time of clotting in the controls in the case of shrimp, starfish, and sperm is probably due to the fact that in these cases the body fluid of fresh *Arbacia* was used. The body fluid of fresh *Arbacia* seems to clot more slowly than that of *Arbacia* kept in an aquarium for four or five days.

These extracts were also injected into the body cavity, and, when the membrane was removed, the fluid had already clotted within the shell. Such clotting does not take place under normal conditions when the membrane is removed very carefully (Pl. I, Figs. 1 and 2).

Certain investigators have described a breakdown of what they call pigment granules imbedded in the jelly of the sand dollar egg when the eggs are broken under pressure. They have also noticed the fusion of these granules at different times. These so-called pigment granules resemble very closely the pigmented cells found in the body fluid of the sand dollar. It seems probable that in reality they are pigmented blood cells that have been caught in the jelly of the egg. The pigment granules of these cells are dispersed throughout the cells in the same manner as they are in the body fluid cells. There also seems to be present a definite nucleus and cell membrane which become even more definite after the pigment granules have broken down. These same cells can be found at various places in the ovaries and testes and are not confined to the egg jelly. When the egg is broken under pressure and the exudate comes in contact with these cells, the pigment granules break down just as the pigment granules of the body fluid cells of *Arbacia* break down when treated with *Arbacia* egg extracts.

Action of K and Ca.—It is well known that the inorganic ions play an important role in physiological processes, and for this reason it was thought advisable to test the effect of some of these ions on the body fluid of the sea urchin.

An isotonic solution of KCl or of any of the K salts appears to have exactly the same effect as the tissue extracts. If 2 cc. of this solution is added to 8 cc. of body fluid, a clot appears within a few seconds and pigment liberation also takes place. This isotonic solution can be diluted twenty times and still be effective (Fig. 3).

TABLE 2

Salt	Average Time of Clotting (Seconds)	Control (Seconds)
0.3 M K_2SO_4	96	3,689
0.53 M KCl	82	3,258
0.53 M KNO_3	68	4,268
0.53 M KSCN	42	3,962
0.3 M $CaCl_2$	324	3,810
0.3 M $BaCl_2$	402	4,319
0.3 M $SrCl_2$	385	3,728

$CaCl_2$ also hastens clotting but not to the same extent as KCl. $BaCl_2$ and $SrCl_2$ appear to have the same effect as $CaCl_2$.

Solutions of 0.53 M KCl and 0.3 M $CaCl_2$ were used as isotonic solutions, although some authors give slightly higher molarity values. However, 0.53 M solutions in the case of monovalent ions and 0.3 M in the case of divalent ions are commonly used as isotonic solutions because in such solutions *Arbacia* eggs neither shrink nor swell.

The K salts appear to be effective in the following order: $SO_4 < Cl < NO_3 < SCN$ (see Table 2). If these solutions are injected into the body cavity, clotting will take place within the shell.

The white or colorless amoeboid cells in the body fluid seem to be composed of granules of the same size and shape as those in the red amoebocytes, and these white granules also appear to break down under the influence of K and Ca.

Action of fat solvents.—Solutions of fat solvents appear to have an accelerating effect on clot formation. These solutions were as effective as the KCl solutions and also brought about pigment liberations. Comparative results are shown in Table 3.

Temperature effects.—Gradual exposure of *Arbacia* to temperatures ranging from 0° C. to 10° C. seems to have a slightly inhibitory effect on clot formation. However, if the body fluid is suddenly placed in a Stender dish which has been cooled to 0° C., the pigment granules break down immediately, and the cells form a clot.

In the same way gradual exposure to high temperatures has no observable effect unless the *Arbacia* is killed by heating. At temperatures above 40° C. the body fluid seems to be permanently altered and will not clot. However, if the body fluid is suddenly placed in a Stender dish which has been heated to a temperature of 40° C., pigment liberation and clotting ensue within a few seconds.

Effect of foreign bodies.—If a piece of boiled intestine or a piece of the membrane or a grain of sand is introduced into the body fluid, the blood cells accumulate around it and, by agglutinating, form a solid mass somewhat similar in appearance to the formation of a thrombus around a foreign body introduced into the blood vessel of a vertebrate. In the case of the sea urchin this thrombus-like mass consists of clotted cells. The cells react in the same way to any foreign body. If any air bubbles are in the fluid, a clot can be seen to form around the outer edges.

TABLE 3

Fat Solvent	Average Time of Clotting (Seconds)	Control (Seconds)
2 per cent ether	56	4,260
5 per cent acetone	66	3,696
0.13 per cent chloroform	48	3,228
2 5 per cent chloral hydrate	72	4,020
2 per cent acetonitrile	42	4,158
5 per cent alcohol	82	2,970

Mechanical stimulation.—If we remove the body fluid from the body cavity with extreme care and then place a drop of this fluid on a slide and examine it microscopically, a marked difference can be noticed between the cells suspended in the plasma and those that have dropped to the surface of the slide. In the former almost all the granules remain unchanged, and no clotting of the cells has taken place. In the latter the cell granules are broken down to a great extent, and clotting has taken place. The mechanical irritation of the slide appears to produce pigment liberation and clotting. If the fluid is stirred with a glass rod, the cells readily adhere to it; and, upon examination, it is found that the pigment granules of such cells have broken down. If some of the cells are placed on a slide under a cover slip, and the water removed from under the cover slip until it exerts a pressure on the cells, the pigment granules can be seen to break down immediately, and cells that are close to one another seem to fuse to form small individual clots (Figs. 4 and 5).

Electrical stimulation.—Passing a direct current through the body fluid appears to hasten clotting considerably and also to bring about pigment liberation. Clotting takes place under these circumstances in about 4 or 5 minutes while the controls clotted in an average time of 35 minutes.

The electrodes were prepared in the following manner: glass tubes of 9-mm. bore were filled at their tips with a 5 per cent agar gel made up in tap water. A saturated solution of $CuSO_4$ was placed over the gel, and the copper wires dipped into this. The body fluid was placed in a 2-inch Stender dish and the electrodes placed at both extremes of the dish. A current of 5 milliamperes was allowed to flow through the fluid until pigment liberation and clotting took place.

Irradiation with ultra-violet light.—The body fluid was irradiated from a Cooper-Hewitt quartz mercury vapor lamp, at a distance from the arc of 28 centimeters. The fluid was exposed for different intervals of time and then examined. Pigment liberation and clotting took place after the cells had been exposed for 4.5 minutes, the controls in this case clotting in an average time of 42 minutes. Longer exposure produced a more profuse liberation of pigment. A single typical clot is not formed under these circumstances, but the cells seem to adhere to one another so as to form a number of small isolated clots.

Effect of hypertonic solutions.—Hypertonic sea water also hastens clotting and brings about pigment liberation. These hypertonic solutions were made by adding 8 cc. of 2.5 M NaCl solution to 50 cc. of sea water. Under the influence of these solutions pigment liberation and clotting took place in about 3 or 4 minutes while the controls clotted on an average of 39 minutes.

Anticlotting agents.—The reaction of the body fluid cells to stimulating agents suggested that perhaps clotting may be prevented by anesthetic agents such as Mg, although the fat solvents reacted in just the opposite manner and hastened clotting. Reagents such as oxalate and citrate which act as anticoagulants in vertebrate blood clotting were also tested.

If an equal amount of a saturated solution of $MgSO_4$ is injected into the body cavity or added to the body fluid as it flows from the shell, clotting will be indefinitely delayed. An isotonic solution of $MgCl_2$ will also inhibit clotting if added in the proportion of about three parts of $MgCl_2$ to one part of body fluid.

If an equal amount of isotonic $MgCl_2$ is added to an isotonic solution of KCl or $CaCl_2$, the K and Ca seem to be antagonized by the Mg and do not cause clotting or pigment liberation. The same is true if an equal amount of isotonic $MgCl_2$ is added to the tissue extract; the tissue extract also seems to be inhibited and does not cause clotting or pigment liberation.

A 3 per cent solution of formalin will also inhibit clot formation. In this case, however, the cells are evidently killed as they move about very feebly or do not move at all. The cells seem to react to this solution in the same manner as when they are heated to high temperatures, i.e., they assume a spherical shape and withdraw their pseudopodia.

A 10 per cent solution of peptone will also inhibit clotting.

Sea water with a pH between 4.2 and 4.6 has a marked inhibitory effect on clot formation but does not prevent it entirely. There are no gross signs of typical clot formation, but small clumps of cells may be observed under the microscope.

If the body fluid is mixed while flowing from the shell with about twice its volume of 0.3 M K-oxalate at pH 7.8, pigment liberation does not take place and clotting is indefinitely delayed. The fluid must be transferred here from the shell to the oxalate very rapidly and must be mixed thoroughly by stirring. The cells not only do not agglutinate to one another but do not adhere to foreign bodies.

Pigment liberation and clotting can also be inhibited by the same process in 0.27 M Na citrate at pH 7.8. In both the oxalate and citrate solutions the cells, both pigmented and nonpigmented, take on a rounded appearance and withdraw their pseudopods.

When body fluid is added as a control to sea water of the same volume as the oxalate and citrate, clotting takes place normally.

If tissue extracts, K salts, or hypertonic solutions are added to oxalated or citrated body fluid, neither pigment liberation nor clotting takes place. Mechanical stimulation, ultra-violet irradiation, or an electric current also have no effect upon material so treated.

The fat solvents, however, do bring about pigment liberation in both the oxalated and the citrated material. Perhaps these reagents are able to penetrate somewhat more deeply into the interior of the cell and cause the ionization of Ca which may be bound there.

DISCUSSION

As a result of these experiments it would appear that normal clotting in the body fluid of *Arbacia* is induced by the liberation of an injury substance when any tissue of the body is damaged. This seems to be evident from the effect of tissue extracts on clotting and from the fact that, if a needle is pushed through the membrane and the inner wall of the test scratched, a clot appears in that region. The effect of this injury substance can be duplicated by K and Ca and certain other reagents. That this substance is not K is evident from the fact that the tissue extract is effective in much higher dilutions than is K. However, from the action of K and Ca on the body fluid, one would be led to venture a suggestion that perhaps both these ions indirectly produce the injury substance.

In some respects clotting of the body fluid of the sea urchin is related to stimulation, in others to clotting as it occurs in vertebrates. Whenever the cells of the body fluid are stimulated either by chemical or by physical means, a breakdown of pigment granules results. Clotting of the cells is always accompanied by this granule breakdown, and any reagent which prevents pigment liberation will also prevent clotting. Leo Loeb (1905), working upon clotted body fluid of *Limulus*, noticed this association and suggested that "it might be very well possible that the same conditions which change the shape and produce the stickiness of cells have also a dissolving influence on the granules." It is also very probable that the breakdown of pigment granules is concomitant with the release of calcium from the surface of the cells as this reaction can be prevented by oxalating or citrating the body fluid. Tissue extract and certain chemical and physical stimulants are evidently able to bring about this release of Ca from the surface of the cell but are prevented from so doing by the presence of oxalate or citrate. All these results appear to be in conformity with the hypothesis developed by Heilbrunn, that a primary effect of stimulation is the release of calcium from combinations located in the periphery of the cell. This release of Ca in the case of the cells of the body fluid seems to be intimately related to clot formation.

It is also interesting to compare the release of pigment from the cells of the body fluid when tissue extracts and KCl are added, with the loss of pigment in *Arbacia* eggs following cytolysis or injury. It is not improbable that there is more than a surface similarity between these two phenomena.

A like phenomenon is also present in the case of the yellow pigment granules in *Arenicola* larvae. The experiments of Lillie (1909) were repeated, and it was found that the same reagents that cause pigment liberation in cells of the body fluid of *Arbacia* also produce pigment liberation in *Arenicola* larvae. After the pigment granules have been broken down, the larvae agglutinate to one another, forming fairly large masses in somewhat the same manner as the body fluid cells agglutinate. Ralph Lillie first described this phenomenon but attributed the release of pigment to an increase in permeability after treatment with certain salts such as chlorides of K, Ca, and Na. This view hardly seems in agreement with the belief that Ca decreases permeability. This release of pigment can also be prevented by oxalating or citrating the larvae. This reaction, therefore, seems to be dependent upon the presence of Ca.

The problem presented here is similar in some respects to the problem upon which so much experimental work has been done in the case of the higher vertebrates. For here too we have a circulatory medium which does not clot while in the animal's body but coagulates whenever the body wall is wounded and the body fluid is in danger of being lost. It seems strange that so little experimental work has been done on this problem in the case of the invertebrates. Evidence derived from more experimental work on the lower forms may very probably throw some light on the amazingly large number of contradictory theories presented as an explanation of blood-clotting in the higher vertebrates. Certainly, there is some similarity between the two forms of clotting. We know that for clotting to take place in the higher forms at least three things are necessary: Ca, the tissue factor, and thrombin under the influence of which fibrinogen is converted into fibrin. In the case of the sea urchin it seems probable that Ca and the tissue factor must also be present before clotting can take place. It seems probable, also, that some thing necessary for clotting may be contributed by the cells. As long as the cells are intact, the body fluid does not clot; but, as soon as the pigment granules break down, clotting takes place. It seems evident that something is liberated from the cells with the breakdown of pigment granules that has effect on clotting. The fact that some substance can be extracted from previously clotted material which will cause clotting in freshly shed body fluid also lends support to this belief. This substance contributed by the cells may be somewhat of the nature of an agglutinin which is present in the cells in an inactive state and is activated by the tissue factor just as thrombin may be activated by the tissue factor in mammalian blood. This agglutinin-like substance may be the "coagulable material" which Schäfer (1883) believes to be responsible for clotting in the sea urchin. Just how these three factors—Ca, the tissue factor, and the agglutinin-like substance—react to cause clotting is somewhat a matter of speculation just as it is a matter of speculation as to how Ca, the tissue factor, and thrombin react to cause clotting in mammalian blood. This problem can be solved only by further experimentation.

More experimental work in the different animal phyla and a comparison of the conditions existing in them may throw some light on the stages through which this property of clotting, so highly exhibited in mammalian blood, was developed in the process of evolution.

SUMMARY

1. The clot is due to cellular elements, and no clotting can occur after these are filtered off.

2. *Arbacia* extracts bring about clot formation when added to the body fluid. Extracts of crustaceans, mollusks, and starfish produce the same effect, some being even more effective than *Arbacia* extracts. *Arbacia* eggs broken up in ground glass or boiled also hasten clot formation. Sperm extract has the same effect. These extracts also bring about a breakdown of the pigment granules.

3. K, Ca, Ba, and Sr also cause clot formation and granule breakdown, K being most effective.

4. Dilute solutions of fat solvents bring about clotting in the body fluid.

5. The addition of a foreign body to the perivisceral fluid will cause a clot to form around it.

6. Hypertonic solutions, electrical stimulation, ultra-violet irradiation, and mechanical stimulation also cause pigment liberation and clotting.

7. $MgCl_2$ prevents clotting and also inhibits the action of any of the agents causing clotting. A 10 per cent solution of peptone will also inhibit clotting.

8. Sea water with a pH between 4.2 and 4.6 has a marked inhibitory effect on clot formation.

9. If the body fluid is thoroughly oxalated or citrated, neither pigment liberation nor clotting takes place regardless of the type of stimulus.

10. Clotting in the perivisceral fluid is normally induced by the liberation of an injury substance when any tissue of the body is damaged.

11. Clotting in the body fluid of *Arbacia* is related in some respects to stimulation, in others to vertebrate blood clottings.

12. The results seem to be in agreement with the hypothesis that, when stimulants act on cells, they cause a release of calcium from combinations located in the periphery of the cell. In the case of the cells of the body fluid, this release seems to be intimately associated with clot formation.

LITERATURE CITED

Costello, D. F. 1932. The surface precipitation reaction in marine eggs. Protoplasma, 17:239.
Cuénot, L. 1891. Etudes morphologiques sur les échinoderms. Arch. Biol., 11:313.
Geddes, P. 1880. Observations sur le fluide perivisceral des oursins. Arch. zool. exper. et générale, 8:483.
Goodrich, E. S. 1919. Pseudopodia of the leucocytes of invertebrates. Quar. Jour. Micro. Sci., 64:19.
Halliburton, W. D. 1885. On the blood of decapod crustacea. Jour. Phys., 6:300.
Heilbrunn, L. V. 1937. An outline of general physiology, p. 80.
Henri, V. 1906. Etude du liquide perivisceral des oursins. Eléments figures. Phénomène de la coagulation et son rôle biologique. Compt. rend. soc. biol., 60:880.
Kindred, J. E. 1921. Phagocytosis and clotting in *Arbacia*. Biol. Bull., 41:144.
———. 1924. The cellular elements in the perivisceral fluid of echinoderms. *Ibid.*, 46:228.
Lillie, R. 1909. On the connection between changes of permeability and stimulation and on the significance of changes in permeability to carbon dioxide. Amer. Jour. Physiol., 24:14.
Loeb, L. 1905. Studies in cell granula and amoeboid movement of the blood cells of limulus. Univ. Pennsylvania, Med. Bull., 18:45.
Nolf, P. 1909. Le Sang des invertébrés contient—il de la thrombine ou les constituants de la thrombine? Arch. internat. de physiol., 7:20.
Schäfer, E. A. 1883. Preliminary notice of an investigation into the coagulation of the perivisceral fluid of the sea urchin. Proc. Roy. Soc. London, 34:370.
Tait, J. 1908. A simple method of observing the agglutination of the blood cells in *Gammarus*. Quar. Jour. Exper. Physiol., 1:247.
———. 1910. Crustacean blood coagulation as studied in the arthrostraca. *Ibid.*, 3:1.
Tait, J., and Gunn, J. 1918. The blood of *Astacus fluviatilis:* a study in crustacean blood, with special reference to coagulation and phagocytosis. Quar. Jour. Exper. Physiol., 12:35.
Theel, H. 1896. Remarks on the activity of amoeboid cells in the echinoderms. Festch. Lilljeborg Upsala, p. 47.

PLATE I

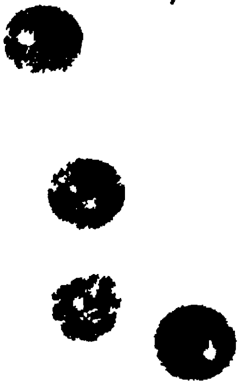
FIG. 1.—Cells before granular breakdown.

FIG. 2.—The same cells in which the granules have broken down after treatment with *Arbacia* shell extract.

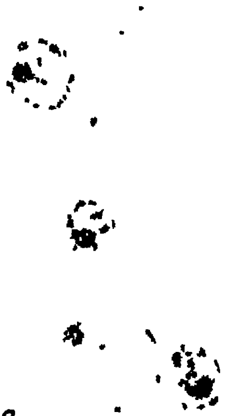
FIG. 3.—Cells undergoing granular breakdown after treatment with KCl. In some of the cells the granules have already broken down, in others they are just about to break down.

FIGS. 4 and 5.—Cells before and after granule breakdown under pressure exerted by the cover slip when water was removed from under it.

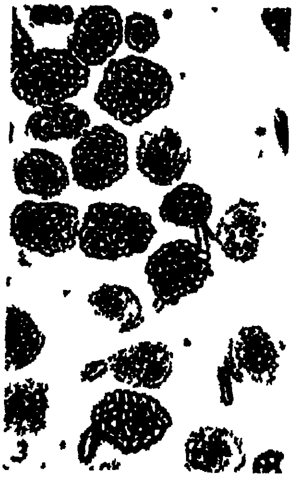
PLATE I



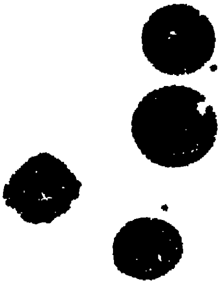
1



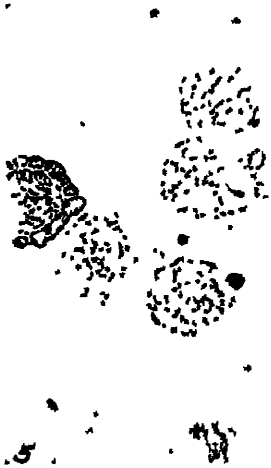
2



3



4



5

ABUNDANT FEEDING FOLLOWED BY RESTRICTED FEEDING AND LONGEVITY IN DAPHNIA[1]

(Four figures)

H. HOWARD DUNHAM
Brown University

VARIOUS studies have been made to determine the effects of underfeeding different animals during the early part of life, followed by abundant feeding for the remainder of life. Studies by Jackson and Stewart (1920) and by Osborne and Mendel (1915) with the albino rat showed that early suppression of growth by limitation in food supply did not cause a loss of ability to resume growth when the diet was made adequate at a later time. A similar study by McCay, Crowell, and Maynard (1935) gave similar results and showed, in addition, that the life-span of male rats was increased when they were given a limited amount of food for a considerable period early in life and were well fed later.

Of chief importance for the study to be reported in this paper is the work of Ingle (1933) and of Ingle, Wood, and Banta (1937), who, using cladoceran material, showed that limitation in amount of food early in life, followed by good feeding for the remainder of life, was an effective method of prolonging the life-span in *Daphnia longispina*. The present paper is concerned with the effect to be obtained by subjecting *D. longispina* to limitation in amount of food at a later period in life after the animals had been well fed during an earlier period.

Cladoceran material is suitable for such experimental work because consideration of hereditary characteristics affecting growth, longevity, and reproduction may be disregarded in view of the fact that *D. longispina* reproduces by diploid parthenogenesis.

MATERIALS AND METHODS

One clone of *D. longispina* was used for all the experiments to be reported here. The animals were taken from Dr. Banta's Line 1284, a line which has been maintained in the laboratory by parthenogenetic reproduction since it was started from animals taken from the wild at Ocala, Florida. All the experimental animals were parthenogenetically produced females. This line is maintained in the laboratory in Banta's (1921) manure infusion medium, and this medium or a dilution of it was used throughout these experiments.

Under ordinary laboratory conditions parthenogenesis is normal, and sexual reproduction occurs only when it is purposely initiated. A young female at birth measures about 0.6 mm. in length, is free swimming, and resembles the adult female except in size and form of brood chamber and condition of ovaries. Under normal conditions (unlimited food and room temperature) the young animal passes through the three juvenile instars in about that number of days. The fourth instar, during which eggs appear in the ovaries, is the adolescent instar. At the beginning of the fifth instar, the

---

[1] The author is indebted to Dr. A. M. Banta for many thoughtful suggestions and much helpful criticism during the course of this work.

first adult instar, the first clutch of eggs passes from the ovaries into the brood chamber, where they are retained during development. At the close of this instar, young are released from the brood chamber and a generation cycle is completed. If culture conditions are favorable, an individual mother may live 18–20 or more instars, produce 200–300 or more young, and attain a maximum size of about 2.8–3.0 mm.

Since the present experiments were concerned with longevity, it was a matter of prime importance to start an experiment with young animals of known age; and it was considered advisable, though not imperative, that all animals used in an experiment should be of the same age. In every series except one, all the animals used had been released within a period of 6 hours. To obtain a large number of animals of the same age, about 150 mothers were taken from the laboratory stock and put separately into 200-cc. wide-mouthed bottles each containing about 100 cc. of manure infusion medium. These stock mothers were kept in an incubator at 25° C. and were transferred to fresh bottles every time they released a brood. The fourth, fifth, or sixth broods produced by these mothers were taken to start a series; the size of such broods was generally over 30, and at the time of their release the mothers appeared to be in prime condition.

In order that all groups of a series be comprised of comparable animals, so far as possible, the young of each brood were distributed uniformly among the various experimental and control groups. Two control groups were provided in each series. One, the normal controls, consisted of mothers cultured throughout life in the normal culture medium containing an abundance of food; the other controls ("semistarved controls") were a group cultured in a medium prepared by diluting this normal medium some thirty to forty times with pond water. Such dilute medium provided a relatively limited amount of food, which permitted animals to grow and reproduce only at a much lower rate than did mothers of the normal control group. Various experimental groups were provided by maintaining a number of animals in normal medium for a definite number of instars and then introducing them into dilute medium at the beginning of a definite instar. At the beginning of a series each animal was placed in a separate 200-cc. wide-mouthed bottle containing about 100 cc. of medium. The bottles were placed in trays and kept in an incubator maintained at a temperature of 23°–26° C. Because there was a difference of about 1° between the top and the bottom of the incubator, it was found advisable to reverse the position of the trays within the incubator from time to time.

Each animal was examined every 12 hours, and such animals as had produced a brood and molted since the preceding observation were measured with a microscope equipped with a micrometer eyepiece before being transferred to a bottle containing a fresh supply of the appropriate medium. The young produced were counted and discarded. All deaths of experimental animals were recorded at these 12-hour intervals.

RESULTS

The results of four complete series of experiments are reported here; they are referred to as Series I, II, III, and IV, according to the chronological order in which they were conducted.

EFFECT ON LONGEVITY

Series I was composed of four different groups, as listed in Table 1, which shows the average longevity for the animals in each group, together with average reproductive life, average number of young per mother, etc. The well-fed controls (normals) had an average life-span of 30.3 days, a figure exceeded by the semistarved ("retarded") controls by about 27 per cent (38.6 days). The normal controls produced, on the average, more than three times as many young as did the retarded controls, and the reproductive index (average number of young per mother per day of reproductive life) was nearly five times as high for normal as for retarded controls. Table 1 also shows that the average longevity for animals well fed to the sixth instar (29.1 days) was only slightly different from the average (30.3 days) for normal controls. But the animals well fed to the beginning of the twelfth instar and then given the same limited food as the retarded controls had a considerably shortened average life-span (25.8 days), as compared with normal controls, the difference (4.5 days) for these two groups being about 15 per cent of the average for normals.

Survival curves (Fig. 1)[2] showing the percentage of each group surviving each day in Series I throw light on certain points not apparent in Table 1. An examination of these curves reveals that animals in the semistarved control group were more variable with respect to longevity than were animals in the normal control group. The same was true for the group well fed to the sixth instar (about the sixth day), as compared with normal animals; but less variability was observed in the group well fed to the twelfth instar (about the eighteenth day). In other words, the slopes of the curves for normals and for animals well fed to the twelfth instar are relatively quite steep, showing that animals in these groups died off at a considerably faster rate than did animals in the other two groups.

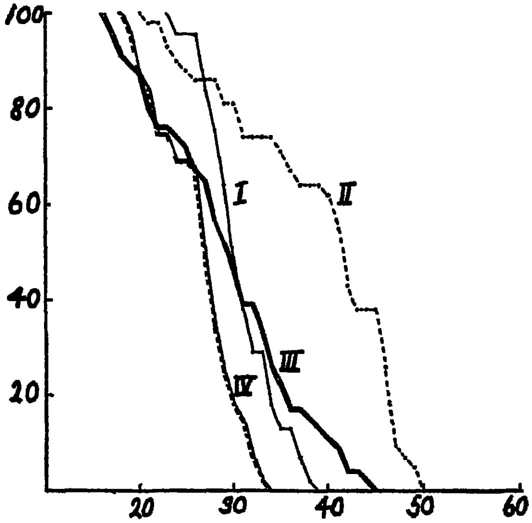


FIG. 1.—Percentage of each group surviving each day, Series I. Ordinates, percentage surviving; abscissas, days of survival. *I* represents well-fed controls; *II*, semistarved controls; *III*, group well fed to sixth instar; *IV*, well fed to twelfth instar.

Table 1 also presents the data for Series II, which was similar in design to Series I except for the inclusion of two additional test groups. The results obtained in Series I had suggested that, if the limited food supply were imposed somewhat earlier than the sixth instar upon previously well-fed animals, an average prolongation of life might be effected. To test this, a group well fed to the beginning of the third instar was included in Series II. Likewise, a group well fed to the beginning of the ninth instar was included, to determine whether at this stage the animals might respond to limited food in about such a manner as did a group well fed to the sixth instar or whether such a group might react to the indicated change more after the fashion of animals deprived of an abundance of food at the twelfth instar. Series II was clearly a long-lived series as compared with Series I.

---

[2] In the accompanying figures retarded controls are the same as semistarved controls.

TABLE 1

DATA ON LONGEVITY AND REPRODUCTION

Group	No. of Mothers	Longevity (Days)	Reproductive Life (Days)	Young per Mother	Reproductive Index	No. of Broods	Young per Brood
Series I							
Normals	45	30.30	24.16	269.40	11.30	11.63	23.11
Semistarved	42	38.62	32.10	74.50	2.40	13.10	5.76
Well fed to sixth instar	46	29.08	23 38	99.46	4.25	10.15	9.80
Well fed to twelfth instar	57	25.77	19 98	165.53	8.28	9.44	17.54
Series II							
Normals	47	36 61	29.66	260.62	8.79	11.47	22.73
Semistarved	34	46.35	36.63	78.44	2.14	14.15	5.54
Well fed to third instar	39	40.52	32.85	69.67	2.12	13.49	5.17
Well fed to sixth instar	38	39.12	32.33	71.21	2.20	12 84	5.54
Well fed to ninth instar	46	28.95	22.10	111.78	5.06	9.22	12.13
Well fed to twelfth instar	51	28.08	20.78	162.04	7.80	8.82	18.36
Series III							
Normals surviving ninth instar	43	33.49	26.62	213.44	8.02	9.98	21.39
Semistarved surviving ninth instar	35	37.16	30 00	99.43	3.31	11.26	8.83
Well fed to ninth instar surviving ninth	33	26.03	19.56	73.30	3.75	5.48	13.36
Graduated ninth to twelfth instar surviving ninth	33	24.32	17.77	66.61	3 75	4.36	15.26
Normals surviving twelfth instar	41	34.30	27.40	219.37	8.01	10.24	21.41
Semistarved surviving twelfth instar	34	37.69	30.54	101.03	3.31	11.44	8.83
Well fed to twelfth instar surviving twelfth	40	28.76	22.08	135.30	6.13	6.95	19.47
Series IV							
Normals surviving ninth instar	48	33.49	26.67	161.42	6.05	9.38	17.22
Semistarved surviving ninth instar	48	36.86	28.69	81.96	2.86	11.17	7.34
Well fed to sixth instar surviving ninth	46	32.07	24.25	98.98	4.08	9.20	10.76
Well fed to ninth instar surviving ninth	48	31.23	24.30	126.25	5.20	9.31	13.56
Graduated ninth to twelfth instar surviving twelfth	39	30.60	23.96	134.26	5.60	9.31	14.42
Normals surviving twelfth instar	40	36.30	29.58	178.33	6.03	10.35	17.23
Semistarved surviving twelfth instar	45	37.90	29.60	84.31	2.85	11.51	7.32
Well fed to twelfth instar surviving twelfth	32	30.33	23.64	118.00	4.99	7.66	15.41

In Series II the semistarved control animals lived 25 per cent longer than normal controls. In this experiment the groups well fed to the third and to the sixth instars gave very similar results and showed a fair increase in longevity over the normal animals. They did not, however, approach the semistarved control group in longevity. The data (Table 1, Ser. II) for the groups well fed to the ninth and twelfth instars are in rather close agreement, especially as regards average longevity. The results obtained here seem to indicate that between the sixth and the ninth instars well-fed *Daphnia,* become physiologically incapable of passing from an environment rich in food into one containing only a limited food supply without suffering a resultant decrease in longevity, whereas until or before the sixth instar they seem physiologically capable of becoming adjusted to the change to the extent that the average longevities of the groups may be increased as compared with normal controls.

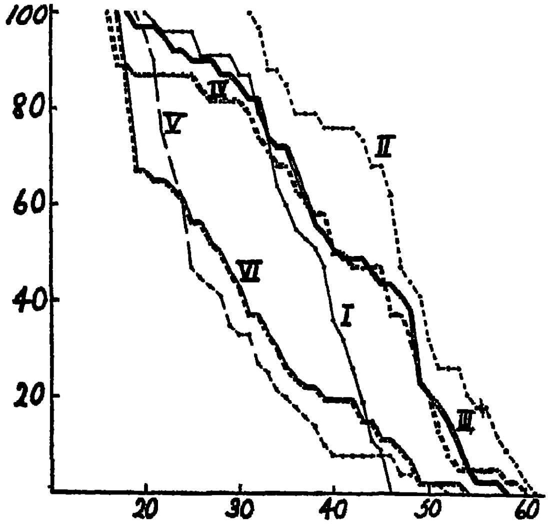


FIG. 2.—Percentage of each group surviving each day, Series II. Ordinates, percentage surviving; abscissas, days of survival. *I* represents well-fed controls; *II*, semistarved controls; *III*, group well fed to third instar; *IV*, well fed to sixth instar; *V*, well fed to twelfth instar; *VI*, well fed to ninth instar.

In comparison with control groups in Series II, there was a high degree of variability with respect to longevity in the groups well fed to the third and to the sixth instars and an intermediate degree of variability in the groups well fed to the ninth and to the twelfth instars. This is shown in Figure 2 where the percentage surviving in each group each day may be read from the survival curves plotted for each group in the series. These curves (for the groups transferred at the sixth, ninth, and twelfth instars—approximately the sixth, twelfth, and eighteenth days, respectively) plainly show that relatively large numbers of animals in the test groups dropped out in a comparatively short time after being changed from normal to dilute medium, but that the animals surviving the change for a few days tended to drop out at a lower rate than was characteristic for controls during the latter part of the experiment.

It will be recalled that a similar result was obtained in Series I with the group well fed to the sixth instar. These considerations led the author to suppose that the very decided decrease in amount of food given these previously well-fed animals in the various test groups at such times as the animals were changed from normal to dilute medium constituted a severe "shock" to the animals which were at such times undoubtedly living at a high metabolic rate, since the normal medium provides approximately optimum conditions. Since considerable numbers in all test groups did survive and since these survivors died off at a lower rate than did normal controls during the latter part of the experiment, it seemed likely that, if normal animals were changed from normal to dilute medium by stages instead of abruptly, animals thus gradually changed might become "conditioned" against the apparent shock. Such an attempt was made in both Series III and Series IV, in which series were included groups as listed in Table 1. In Series III the "graduated" group was arranged by putting a group of normal animals into one-tenth normal medium at the beginning of the ninth instar; into one-twentieth normal at the tenth; into one-thirtieth normal at the eleventh; and finally into one-fortieth normal at the beginning of the twelfth instar. The dilution used throughout for retarded controls in this series was one-fortieth the normal strength.

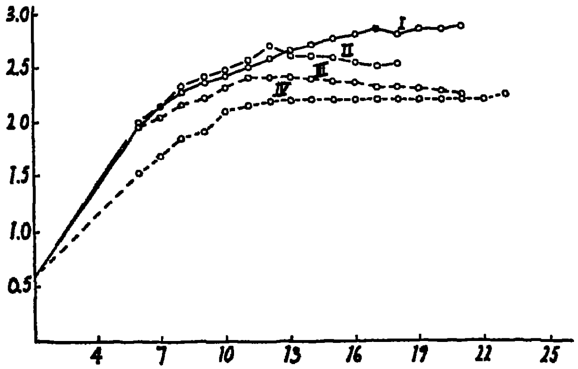


FIG. 3.—Average length of animals in each group at birth and at beginning of each instar after fifth, Series I. Ordinates, body length in millimeters; abscissas, number of instar. *I* represents well-fed controls; *II*, group well fed to twelfth instar; *III*, well fed to sixth instar; *IV*, semistarved control group.

This attempt to condition animals against a sudden change of medium failed, as is evident from an examination of Table 1 (Ser. III). It seemed possible that the earlier steps of the graduating process should be smaller, since one-tenth the normal medium had seemed to have as damaging an effect as did the one-fortieth normal into which the group well fed to the ninth instar was changed abruptly. In Series IV another attempt at conditioning was made, the same as in Series III except that the steps used were one-half normal, one-fourth normal, one-tenth normal, and one-fortieth normal. Table 1 (Ser. IV) shows that this attempt was again futile and that the "conditioned" animals were in most respects about equal to animals well fed to the ninth or twelfth instars and then semistarved.[3]

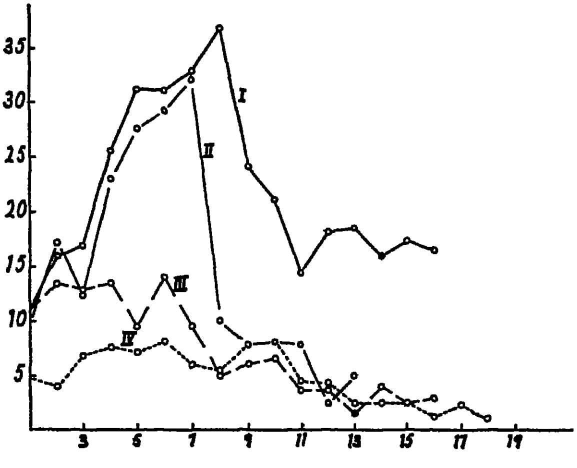


FIG. 4.—Average numbers of young per brood for all groups, Series I. *I* represents well-fed control group; *II*, group well fed to twelfth instar; *III*, well fed to sixth instar; *IV*, semistarved control group. Ordinates, number of young in brood; abscissas, number of brood.

In both Series III and IV large numbers of animals in the groups well fed to the ninth and to the twelfth instars died during the first instar after the change from normal dilute medium. This was also true for the graduated group. For this reason no animals in these groups were considered in the data except those which survived one complete instar after the change. For purposes of comparison the data for control groups was treated similarly in these two series.

---

[3] In the author's opinion neither Series III nor Series IV is very significant, since some difficulty with culture medium was experienced in both series. Even normal controls in these two series were atypical in average longevity, etc., as compared with normal controls in Series I and Series II or as compared with normal animals reared by other workers in this laboratory.

BODY GROWTH

The growth of all groups in each series was studied; but, since growth phenomena were much the same in all series, only the data for growth of the four groups studied in Series I will be considered in this paper. The average length of the animals at birth in all four groups was slightly over 0.6 mm. Growth was fairly rapid for all groups until the beginning of the sixth instar, although there was a considerable difference between the growth of animals being maintained in normal medium and the growth of the semistarved control group. As can be seen from Figure 3, the average maximum length attained by the normal animals was slightly over 2.8 mm., a size not attained until the beginning of the sixteenth instar. From the beginning of the twelfth instar the semistarved controls quite regularly measured around 2.2 mm. Neither the group well fed to the sixth instar nor the group well fed to the twelfth instar attained as great an average maximum length (2.4 mm. for the former group, and 2.7 mm. for the latter) as that reached by normal controls. The decrease in average length toward the end of the experiment shown for the test groups in Figure 3 may in part be ascribed to the fact that larger individuals in the two groups had a tendency to die earlier than did smaller ones, but in addition an appreciable number of individuals in these groups showed a positive decrease in length for 1 or 2 instars before death.

SIZE OF BROODS

The wide difference between the average number of young per brood for normal controls and for semistarved controls is apparent in Figure 4. Naturally, the group well fed to the twelfth instar was rather close to the normal controls until the period of starvation began, as was true also of the group well fed until the sixth instar. After the animals in these two groups had been transferred into the dilute medium, they promptly approached a level of reproductive activity about equal to that characteristic of the semistarved control group.

DISCUSSION

Ingle, Wood, and Banta (1937) showed that *Daphnia* which had been semistarved up to the beginning of the twelfth, fifteenth, or even eighteenth instars were physiologically younger animals than normal controls at the same instars, as was apparent from their prompt resumption of rapid growth, increased reproductive activity, and increased longevity. They continued to live for a singificantly long time after both control groups had died. But the results reported here indicate that, when the opposite sort of treatment (well fed earlier and semistarved later) was imposed upon *Daphnia*, only those animals subjected to environments poor in food from the beginning of the third or sixth instars were young enough to be able to adjust themselves to the lowered metabolic rate enforced by such an environment so as to live longer than normal control animals. Animals well fed to the ninth or twelfth instars and then semistarved suffered severely from lack of food, as was evident from the immediate, sharply increased mortality, the marked decline in numbers of young produced, the later negative growth in many individuals, and a somewhat shorter life-span as compared with normal controls.

Well-fed animals undoubtedly age rapidly, for they grow and reproduce at a comparatively high rate. Perhaps the fact that they have aged considerably by the ninth or twelfth instars partly explains the fact that some of them are incapable of becoming adjusted to living at a lower rate of metabolism and thus fail to survive very long when given only a limited amount of food. Such an interpretation leaves unexplained the fact that in some series a number of individuals survived after the change from normal to dilute medium for so long a time as to outlive the longest-lived, normal controls.

Another point to be borne in mind is that animals well fed to the ninth or twelfth instars were considerably larger at these instars than were semistarved controls at these same instars, which, of course, means that these larger animals were composed of a greater mass of protoplasm and required more nourishment. Since they received the same treatment as semistarved controls after the beginning of the ninth or twelfth instars, they must have received less food in proportion to their size than did semistarved controls; hence, they were really more severely starved. A simple calculation based on body-length data taken from Figure 3 will serve to show approximately how great was the size difference at the beginning of the twelfth instar between the animals in the group well fed to the twelfth instar and those in the semistarved control group. The average length of the animals in the former group at this stage was 2.6 mm., while the average length for the latter group was 2.2 mm. In other words, the former figure is to the latter as the ratio 1.18:1.0. When this ratio is cubed, it appears that the average size of the well-fed group at this stage was approximately 1.64 times as great as the average size of the semistarved control animals. It seems reasonable that animals so much larger than semistarved control animals might be rather severely starved while semistarved control animals would not suffer from lack of food; furthermore, the latter animals were already adjusted to the dilute medium.

SUMMARY AND CONCLUSIONS

Longevity of *D. longispina* has been studied for groups of animals well fed to the beginning of the third, sixth, ninth, and twelfth instars and then provided with food in limited amounts (semistarved). Results for these groups have been compared with results for controls well fed throughout life and with results for controls semistarved throughout life. Reproductive activity and growth were also studied for the various groups

The data indicate that, when *Daphnia* are well fed to the beginning of the third instar and then semistarved, the average longevity of the group is about 10 per cent higher than the average for well-fed controls but about 13 per cent lower than for semistarved controls. When *Daphnia* are well fed to the sixth instar and then semistarved, the average longevity of the group is near that for normal controls; and when well fed to the ninth or twelfth instars and then semistarved, the average longevity is lower than for well-fed controls. However, greater variability with respect to longevity is usually observed in these various test groups than is observed in a normal, control group; but maximum length attained and average numbers of young produced per mother are always lower in the test groups than in the well-fed, control group

It seems to make little or no difference whether well-fed animals are changed suddenly from normal to dilute medium or are introduced into the dilute medium by stages.

The suggestion is made that animals well fed to the ninth or twelfth instars and then given a limited supply of food show a decreased average longevity as compared with normal controls because they have aged considerably by the time the change is made and are therefore incapable of becoming adjusted to a scanty supply of food. It is also pointed out that animals well fed to the ninth and twelfth instars are about 1.6 times as large as semistarved controls at the same stages. Thus, when well-fed animals are given the same food as semistarved controls at these stages, they are in reality starved more severely than are the controls with which they are compared.

The foregoing results, together with those of Ingle *et al.*, indicate that *Daphnia* have relatively short lives if well fed for 9 or more instars and then given only a limited food supply; that *Daphnia* well fed throughout life (normal controls) stand next in order of longevity; that *Daphnia* continuously maintained in limited food live longer than continuously well-fed animals; and that animals maintained in limited food for 9, 12, 15, or even 18 instars and then given abundant food show even greater longevity in direct proportion to the number of instars so maintained.

LITERATURE CITED

BANTA, A. M. 1921. A convenient culture medium for daphnids. Science, N.S., 53:557.

INGLE, L. 1933. Effects of environmental conditions on longevity. Science, N.S., 78:511.

INGLE, L.; WOOD, T. R.; and BANTA, A. M. 1937. A study of longevity, growth, reproduction, and heart rate in *Daphnia longispina* as influenced by limitations in quantity of food. Jour. Exper. Zoöl., 76:325.

JACKSON, C. M., and STEWART, C. A. 1920. The effect of inanition in the young upon the ultimate size of the body and of the various organs in the albino rat. Jour. Exper. Zoöl., 30:97.

MCCAY, C. M.; CROWELL, M. F., and MAYNARD, L. A. 1935. The effect of retarded growth upon the length of life span and upon the ultimate body size. Jour. Nutrition, 10:63.

OSBORNE, T. B., and MENDEL, L. B. 1915. The resumption of growth after long continued failure to grow. Jour. Biol. Chem., 23:439.

STUDIES IN MASS PHYSIOLOGY: EFFECT OF NUMBERS UPON THE OXYGEN CONSUMPTION AND LOCOMOTOR ACTIVITY OF CARASSIUS AURATUS[1]

ARTHUR SHLAIFER
Whitman Laboratory, University of Chicago

RESEARCH on animal aggregations has shown that many physiological processes of animals are controlled or modified by the number of animals mutually participating in these processes. Allee (1931 and 1934) has comprehensively reviewed the work that has been done in this field. "Group effects" have been found for breeding, hibernation, estivation, growth, death-rate, resistance to toxic agents, and other processes. An effect of grouping on oxygen consumption has been demonstrated by Allee (1927), Schuett (1933 and 1934), and Bowen (1932).

Schuett (1933), attempting to bridge the gap between the solitary fishes and those which exhibit highly integrated mechanisms of aggregation, reported that, in a given volume, the oxygen consumption of isolated goldfishes was significantly higher than if in a group of four. Stating that the unmodified Winkler technique he had used for determining oxygen tension was at fault, owing to nitrite contamination, Schuett (1934) repeated his experiments, using the permanganate modification of the Winkler method, and found no significant difference in oxygen consumption between goldfishes in isolation and in groups of four. Schuett (1934) and Escobar, Minahan, and Shaw (1936) pursued the analysis of the problem by determining the effect of grouping on the locomotor activity of goldfishes; they found a significantly higher rate of activity if the animals were isolated than if they were grouped.

It has been the purpose of the present studies to repeat the analysis of this problem and to find the correlation, if any, between oxygen consumption and locomotor activity.

MATERIALS AND METHODS

The goldfish, *Carassius auratus* Linné, was the experimental animal throughout.

The oxygen consumption was determined by means of the modified Winkler technique, as described by Allee and Oesting (1934). The permanganate modification was used to eliminate the error due to nitrite contamination. Samples of water were taken immediately before and after the test period. These were analyzed for oxygen in terms of cubic centimeters of dissolved oxygen per liter of water. The difference, multiplied by the number of liters of water used and divided by the number of fishes in the group, yields the oxygen consumption per fish.

Heavy mineral oil was used as a seal to prevent the leakage of oxygen from the air into the water during the course of the experiments. The oil seal was approximately 3.5 cm. thick. There is the criticism that mineral oil is not a perfect seal and that oxygen will diffuse through into the water and raise the tension. Control experiments show that this is true. The controls were run with oil that had been used from one to seven times

---

[1] The writer wishes to express his gratitude to Professor W. C. Allee for the suggestion of this problem and for his helpful advice and criticism.

during a period of 2½ weeks. Fishes in isolation, in groups of two, and in groups of four were allowed to bring down the dissolved oxygen content of the water to the average found at the end of the experiments on fishes in these respective groupings. This assures starting with the usual lower tensions used; and the leaks found are nearly or quite the maximum, not minimum, possible under the given conditions, since the oxygen leak is the greater (other things being equal) the lower the amount of dissolved oxygen in the oil-protected water. Results show a leakage range of from 1.15 per cent to 1.55 per cent of the total oxygen available in both 6 and 12 liters in 3 hours. The greatest leakage occurs at tensions found at the end of the experimental period when four fishes are present. This means that the apparent oxygen consumption of the group of four has been decreased by the leak more than that of the group of two or of the isolated fishes. However, the actual difference in oxygen leaks of the various groups is almost negligible and is not sufficient to alter the conclusions reached in these experiments.

The fishes for each day's tests were picked at random from a lot of twenty selected so as to be approximately equal in size. Their length was between 8 and 10 cm. from the tip of the nose to the base of the tail. Animals of this rather large size were used because (1) one such fish consumes a measurable amount of oxygen in 3 hours; (2) a 3- or 4-hour experimental period is less subject to variations—light intensity, for example—than is a longer period; and (3) the shorter the test period the less the oxygen leak through the oil.

Rectangular, assembled aquariums whose maximum capacities were 7.5 and 15 liters of water were used, the smaller when the experimental volume was 6, the larger when the experimental volume was 12 liters of water in all sets of experiments but two, in which the volume was only 6 liters. The dimensions in centimeters of these aquariums were:

Liters	Length	Width	Depth
7.5	25	20	13 (to the 6-liter mark)
15	30	27	8 5 (to the 6-liter mark) 17 (to the 12-liter mark)

Experiments A, B, C, and D on oxygen consumption were performed at the Hull Zoölogical Laboratory between the hours of 10:00 A.M. and 4:00 P.M. Well water was used at all times. Since the laboratory faces the north, the variations in light intensity were reduced to a minimum during the experimental hours. The temperature range was from 20° to 22° C. Experiments E, F, G, and H on oxygen consumption were performed at the near-by Whitman Laboratory. Since well water was not available, lake water, from which the chlorine and sediment had been removed by filtering through activated charcoal and limestone, was used. Temperature and illumination conditions were approximately the same as at the Hull Laboratory. All experiments performed at Whitman Laboratory were carried out between 12:30 P.M. and 5:00 P.M. The fishes were fed once a week, at the conclusion of a day's experiments, and were not used for at least 20 hours thereafter.

At the end of each day's experiments, the animals were placed into a common aquarium, from which they were picked at random to be placed in isolation, in groups of two, and in groups of four into aquariums containing fresh water through which air was constantly bubbled. These aquariums contained the same volumes of water as were used in the tests the next day. A 20-hour period of acclimatization was allowed in experiments A, B, C, and D and a 21-hour period in Experiments E, F, G, and H. Immediately before each day's tests the air tubes were removed, the water was again changed (except in the "conditioned"-water experiments), and the aquariums were filled 1 liter above the mark. The extra liter of water was added to replace that lost in the process of obtaining three samples of water for analysis at the beginning of the experimental period. These samples were obtained immediately after placing a 3.5-cm. layer of oil on the water. At the close of the test period, samples were again taken and analyzed.

The experiments on locomotor activity were all performed at the Whitman Laboratory under the general conditions mentioned above. The volumes of water, the aquariums, and the feeding and acclimatization techniques were the same as those used in the oxygen-consumption experiments. Also, a 3.5-cm. layer of oil was placed on the water in order to duplicate more completely the general conditions of the experiments on oxygen consumption. In the activity tests the oxygen leak does not enter as a direct factor of error because activity is measured by the observer, not indirectly by chemical analysis. From the twenty fishes used in the oxygen-consumption experiments a group of seven in Experiments A, B, and C and a group of nine in Experiments D, E, F, and G were selected; all the animals selected had distinctive color patterns, so as to be readily identified, and were of approximately the same length.

The technique employed in measuring locomotor activity was that described by Escobar, Minahan, and Shaw (1936) except that all observations were made by one person. Aquariums of the dimensions and capacities listed above were marked off with red India ink into 5-cm. squares on all four sides. Since not all of the dimensions of the aquariums were multiples of five, some of the boxes were rectangles rather than squares. However, movement of a fish into one of these smaller boxes was considered equivalent to movement into the larger squares, since the variable was the same for the various types of grouping. The 5-cm. squares, when viewed in space, constitute a series of imaginary cubes of 125 cc. capacity through which the animals pass when swimming. The eye of the fish was used as the anatomical landmark in judging whether it had passed from one cube into another. The swimming of a fish from one cube into another constitutes a "movement."

There are several sources of error inherent in this method. The greatest lies in the poor estimation of the number of cubes traversed by a rapidly moving animal. When it is moving in several planes at once diagonally across the aquarium, the error is increased. The error can be diminished to some extent by having the guide lines as narrow as possible without obscuring vision.

Another source of error is the fact that fishes may make several movements within a cube; these movements are not recorded in the count. Or they may, by one strong movement, traverse as many as five cubes; and we have no data concerning the oxygen requirements of relatively strong and relatively weak movements which move the animal through the same space. Also, in moving horizontally or vertically from one cube into another, a fish may traverse as many as 9 cm. or as little as 1 cm.

No attempt has been made to differentiate vertical, horizontal, and diagonal movements. The observer is stationed about 2 feet away from the aquarium. It is essential that no sudden noises or movements occur during the observations, as the animals may react violently to them. At no time during the recorded experiments did the fishes appear to be reacting to such external stimuli. Neither did they give indications of being aware of the presence of the observer. With some practice and the aid of two mirrors placed so as to reflect the narrow sides, the observer facing the long side of the aquarium with his eyes below the level of the water can observe movements in three dimensions, though the accuracy is undoubtedly less than with three observers.

Since the fishes were of distinctive color patterns, individual records could be kept. Each animal was observed for 5 minutes of each experimental hour. Each was placed in a different type of grouping each day so that possible variations might be more evenly distributed. Time was recorded by a stop watch.

OXYGEN CONSUMPTION

Oxygen tension is an important factor in experiments involving oxygen consumption of aquatic animals that utilize dissolved oxygen, and three general categories are recognized (Hyman, 1929): (1) animals completely dependent on the oxygen tension of the water (the lower the tension the less the respiratory rate; the higher the tension the greater the respiratory rate); (2) animals independent of the oxygen tension at the median ranges and dependent at the extremes; and (3) animals independent of the oxygen tension of the medium. Keys (1930) says that no general statements can be made for fishes and that at the asphyxial level their oxygen consumption is very low. Toryu (1927) concludes that *C. auratus* is independent of the oxygen tension of the water between 7.5 and 2 cc. of oxygen per liter. This would indicate that it belongs in class (2).

By correlating the oxygen tension at the end of an experiment with the total oxygen consumption of the fishes, it was possible to determine that the animals in the present set of experiments were independent of the oxygen tension from 7.0 to 1.5 cc. of oxygen per liter. Below this the oxygen-consumption rate was lower and drifted with the tension. The results of the oxygen-consumption experiments listed are those in which the oxygen tension at the conclusion of the experiment was 1.5 cc. per liter or higher.

In Experiments A–D of Table 1, each figure for the oxygen consumption per fish in a group of four is an average of the consumption of the four animals in that experiment. Similarly, the consumption per fish in a group of two is the average of the consumption of the two fishes. Each figure for the oxygen consumption of an isolated animal does not represent an average of four or of two fishes, each in isolation, but is the observed respiration by one. Hence, Experiments A–D in Table 1 do not represent an equal sampling. The fishes in a group of four have had the greatest sampling, and those in isolation the least. Experiments E–H of Table 1 do have equal sampling, since there are four times as many cases listed for isolated fishes as for those in a group of four and twice as many as for those in a group of two.

Tables 1 and 1*a* summarize the effect of grouping on the oxygen-consumption of the goldfish. In general, these tables indicate that isolated fishes consume more oxygen than do those in a group of two or four, and that fishes in a group of two tend to consume the same amount of oxygen as do those in a group of four. In some cases, e.g., B and F in the "I vs. II" column and H in the "I vs. IV" column in Table 1*a*, the differences lack statistical significance. Conditions B and C in the "II vs. IV" column of Table 1*a* indicate that fishes in a group of two have a significantly higher oxygen-consumption rate than do animals in a group of four. The combined experimental means show very good significance for the "I vs. II" and "I vs. IV" relationships, while the "II vs. IV" relationship shows no statistical significance, though the *P* value is close to the upper limit of accepted statistical significance.

TABLE 1

EFFECT OF NUMBERS PRESENT UPON THE AMOUNT OF OXYGEN CONSUMED PER FISH BY GROUPED AND ISOLATED GOLDFISHES UNDER EXPERIMENTAL CONDITIONS

EXPERIMENTAL CONDITION	TEST PERIOD (HOURS)	I. ONE FISH		II. TWO FISH		IV. FOUR FISH	
		No. of Cases	Mean Cc. O_2 Consumed per Fish	No. of Cases	Mean Cc. O_2 Consumed per Fish	No. of Cases	Mean Cc. O_2 Consumed per Fish
A[1,3,4,6]...........	4	12	9.21	15	5.67	10	4.64
B[1,4,6]...........	4	18	8 46	19	8.43	8	5.80
C[1,5,6]...........	4	20	9.45	20	7.28	11	5 69
D[2,5,6]...........	4	34	12.36	34	8.17	24	8.24
E[1,4,6]...........	3	20	7 85	10	5.82	5	5.07
F[2,5,6]...........	3	20	8 76	10	7 02	5	6.18
G[1,4,7]...........	3	12	6.50	6	3.91	3	3.72
H[2,5,7]...........	3	12	7.54	6	3.54	3	4.15
Average of combined means.	....	8	8.77	8	6.23	8	5 44

Six liters of water.

[2] Twelve liters of water.

[3] Preliminary set.

[4] In 7.5-liter aquarium.

[5] In 15-liter aquarium.

[6] Unconditioned water.

[7] "Conditioned" water.

TABLE 1*a*

STATISTICAL ANALYSIS OF TABLE 1 SHOWN IN *P* VALUES*

Experimental Condition	I vs. II	I vs. IV	II vs. IV
A..................	.0028	.0020	.0800
B..................	None†	.0476	.0044
C..................	.0002	.0000	.0003
D..................	.0004	.0000	None†
E..................	.0148	.0160	.1216
F..................	.0640	.0370	.4000
G..................	.0040	.0250	.6620
H..................	.0010	.0950	.4780
A–H combined‡..	.0012	.0000	.0566

* Upper limit of significance is set at .05. This is three times the probable error. A *P*-value of .0010 indicates good significance, while a value of .1000 indicates no significance ("Student," 1925).

† "None" indicates that no significance was calculated, since the means are almost identical.

‡ In the combination of A–H in Table 1*a* and A–G in Table 2*a* it must be noted that in some cases the oxygen consumption and locomotor activity of the fishes are significantly affected by differences in the quantity and quality of the water (see Tables 4–9). However, the combinations are justified since we are dealing with sets of paired data.

LOCOMOTOR ACTIVITY

Tables 2 and 2*a* summarize the effect of grouping on the locomotor activity of the goldfish. The general technique used has been described above. There has been equal sampling for the fishes in each type of grouping, since individual records have been kept. Each animal has been in each type of grouping, in any given experiment, for the same period of time. The test periods were 3 and 4 hours, with each animal under observation for 5 minutes of each hour of that time.

TABLE 2

EFFECT OF NUMBERS PRESENT UPON THE ACTIVITY OF GROUPED AND ISOLATED GOLDFISHES UNDER EXPERIMENTAL CONDITIONS (RESULTS ARE IN TOTAL AMOUNT OF MOVEMENT* PER FISH PER 5-MINUTE PERIOD OF OBSERVATION)

EXPERIMENTAL CONDITION	TEST PERIOD (HOURS)	I. ONE FISH		II. TWO FISH		IV. FOUR FISH	
		No. of Cases	Mean Activity	No. of Cases	Mean Activity	No. of Cases	Mean Activity
A[1, 3, 5, 7]	4	7	82.9	7	48.0	7	54.8
B[1, 4, 5, 7]	4	7	55.0	7	47.2	7	41.8
C[2, 4, 5, 7]	4	7	76.7	7	48.9	7	45.4
D[1, 3, 5, 7]	3	9	137.8	9	76.1	9	74.9
E[2, 4, 5, 7]	3	9	154.0	9	76.7	9	65.7
F[1, 3, 6, 8]	3	9	136.8	9	81.1	9	61.5
G[2, 4, 6, 8]	3	8	163.5	8	78.3	8	73.9
Average of combined means	....	7	115.2	7	65.2	7	59.7

* See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement.'

[1] Six liters of water.

[3] In 7.5-liter aquarium.

[5] Unconditioned water.

[2] Twelve liters of water.

[4] In 15-liter aquarium.

[6] "Conditioned" water.

[7] Each case is the average of twelve 5-minute observation periods.

[8] Each case is the average of six 5-minute observation periods.

TABLE 2*a*

STATISTICAL ANALYSIS OF TABLE 2 SHOWN IN *P*-VALUES*

Experimental Condition	I vs. II	I vs. IV	II vs. IV
A	.0170	.0110	.2200
B	.3700	.0094	.3560
C	.0390	.0228	.3700
D	.0204	.0102	.8770
E	.0400	.0220	.1964
F	.0820	.0266	.0758
G	.0678	.0454	.6160
A–G combined†	.0030	.0032	.1700

* See Table 1*a* for discussion of *P*-value.

† See Table 1*a* for discussion of combination of experimental means.

The experiments on locomotor activity were performed to see whether the "group effect," as manifest in the oxygen-consumption experiments, is correlated with the amount of movement performed by each fish. Since, in general, oxygen consumption is directly proportional to activity, the locomotor-activity experiments should give results similar to those on oxygen consumption.

Tables 2 and 2*a* indicate, in general, that isolated fishes have a higher rate of locomotor activity than do those in a group of two or a group of four, and that fishes in a group of two tend to have the same rate of locomotor activity as do those in a group of four. Thus, the results correlate, as they should, with the results on oxygen consumption. In several cases, e.g., B, F, and G in the "I vs. II" column of Table 2*a*, the differences lack statistical significance. The combined experimental means show good significance for the "I vs. II" and the "I vs. IV" relationships, and a *P*-value considerably above the upper limit of accepted statistical significance for the "II vs. IV" relationship.

Since these observations on locomotor activity were made over a period of 3 or 4 hours with an oil seal, and since no Winkler analyses were made for oxygen tension at the end of the test period, the criticism might be made that the fishes in the group of four might have been reacting to an oxygen tension below 1.5 cc. per liter during the latter part of the test period. If so, the results obtained could be attributed not to a "group effect" but to a low oxygen tension which inhibits respiration and activity.

TABLE 3

COMPARING THE ACTIVITY OF FISHES IN A GROUP OF FOUR DURING THE FIRST AND LAST HOURS OF THE EXPERIMENTAL PERIOD (RESULTS ARE IN TOTAL AMOUNT OF MOVEMENT* PER FISH PER 5-MINUTE PERIOD OF OBSERVATION)

EXPERIMENTAL CONDITION†	TEST PERIOD (HOURS)	FIRST HOUR		LAST HOUR		*P*-VALUE¶ FIRST VS. LAST
		No. of Cases	Mean Activity	No. of Cases	Mean Activity	
A‡	4	7	66.7	7	51.0	.0840
B‡	4	7	44.0	7	37.2	.1800
C‡	4	7	49.7	7	45.0	.4680
D§	3	9	77.1	9	75.2	None**
E§	3	9	64.1	9	63.1	None**
F‖	3	9	65.0	9	55.8	.2480
G‖	3	8	70.9	8	72.2	None**
Average of combined means††	....	7	62.5	7	57.1	.0348

* See discussions of locomotor activity under "Materials and Methods" for explanation of term "movement."

† Letters correspond to those in Table 2.

‡ Each case is the average of three 5-minute observation periods

§ Each case is the average of four 5-minute observation periods.

‖ Each case is the average of two 5-minute observation periods.

¶ See Table 1*a* for discussion of *P*-value.

** "None" indicates that no significance was calculated, since the means are almost identical.

†† See Table 1*a* for discussion of combination of experimental means.

Accordingly, the movements of fishes in a group of four during the first hour of the test period, when the oxygen tension is the highest, were compared with the movements during the last hour of the test period, when the oxygen tension is the lowest. The results are summarized in Table 3.

In no test was the activity of the fishes significantly higher during the first hour of the test period. However, a consideration of the combined means does show a significantly higher rate of activity during the first hour, as compared with that of the last hour. When, however, this is compared with the larger mean differences in Table 2, it can be seen that the factor of inhibiting oxygen tensions is not the principle one at work. In fact, under the conditions of these experiments, it is reasonable to assume that the lower activity during the last hour of the test period indicates a more complete recovery from the effect of handling incident to manipulations near the beginning of the observations, rather than being primarily a result of lessened oxygen supply.

When isolated fishes and those in a group of four are compared, the "group effect" on their oxygen consumption and locomotor activity is apparent. Isolated animals are more active and have a higher rate of oxygen consumption. The only exception to each individual comparison being significant when considered alone is found in H of Table 1*a*, and there, we believe, the lack of significance is due to the small number of tests for the group of four. Supporting this view is the marked significance when A–H are combined.

The group of two seems to occupy the same place as the group of four in the comparison of isolated and grouped fishes. There are more cases where individual experiments fail to show that the higher oxygen consumption and locomotor activity of the isolated animal is significant when compared with a fish in a group of two, but the combined means of all the experiments indicate good statistical significance. In general, the fishes in groups of two tend to have the same rate of oxygen consumption and locomotor activity as those in groups of four. Such results may indicate that the isolated state in the phenomenon is a unique one.

VOLUME RELATIONS

Two important factors that enter into the analysis of the problem are the volume of water available to each animal and the dimensions of the container. Schuett (1933) states that when the volume of water per fish is equal the "group effect" on oxygen consumption disappears. Escobar, Minahan, and Shaw (1936) state that "when a fish is limited to a given volume of water the configuration of that volume or the interrelation between the relative magnitudes of its three dimensions determines to a considerable extent the total amount of movement." They find that when goldfishes in isolation and in groups of four are placed in four different volumes of water—1,625 cc., 3,250 cc., 4,875 cc., and 6,500 cc.—minimal activity in grouped and isolated animals occurs at a volume of 4,875 cc., having a depth of 16.92 cm., a width of 12 cm., and a length of 24 cm. The width and length are constant for all volumes. "The locomotor mechanisms of fishes (with a few exceptions, e.g., seahorse) are adapted to propel the fish along the long axis of its body, the latter being normally oriented in most species of fishes in a horizontal plane," say Escobar, Minahan, and Shaw (1936).

Two questions involved in the volume-relationship analysis are: (1) Does the oxygen consumption and locomotor activity of a goldfish vary with the volume of water available to it? (2) When the volume of water per fish is equal, does the "group effect" on oxygen consumption and locomotor activity disappear? Tables 4–7 attempt to answer these questions, utilizing the data in Tables 1 and 2. Only those data were used with which legitimate paired comparisons could be made.

Table 4 indicates that, in the cases tested, a goldfish has a higher rate of oxygen consumption when in a larger volume of water. Table 6, the corresponding table on locomotor activity, should confirm the results of Table 4 but does not. An increase in volume does not produce an increase in the activity of a goldfish. This contradiction

TABLE 4

COMPARING THE OXYGEN CONSUMPTION OF FISHES IN ISOLATION, GROUPS OF TWO, AND GROUPS OF FOUR IN 6 LITERS WITH THE OXYGEN CONSUMPTION OF SIMILAR COMBINATIONS IN 12 LITERS*

SIX LITERS		TWELVE LITERS	
Grouping	Mean Cc. of O_2 Consumed per Fish	Grouping	Mean Cc. of O_2 Consumed per Fish
Isolated..........	8.46 7.85 6.50	Isolated.........	12.36 8.76 7.54
Group of two	8.43 5.82 3.91	Group of two....	8.17 7.02 3.54
Group of four.....	5.80 5.07 3.72	Group of four....	8.24 6.18 4.15
Mean........	6.17	Mean.......	7.33

Statistical significance of the difference, .0236

---

* Figures are based on data in Table 1.

TABLE 5

COMPARING THE OXYGEN CONSUMPTION OF FISHES IN ISOLATION OR GROUPS OF TWO IN 6 LITERS WITH THAT OF FISHES IN GROUPS OF TWO OR FOUR IN 12 LITERS WHEN THE VOLUME OF WATER PER FISH IS EQUAL*

SIX LITERS		TWELVE LITERS	
Grouping	Mean Cc. of O_2 Consumed per Fish	Grouping	Mean Cc. of O_2 Consumed per Fish
Isolated..........	8.46 7.85 6.50	Group of two....	8.17 7.02 3.54
Group of two.....	8.43 5.82 3.91	Group of four....	8.24 6.18 4.15
Mean........	6.83	Mean.......	6.22

Statistical significance of the difference, .2838 makes a generalization impossible but may mean that, as used, the oxygen-consumption tests are the more sensitive.

---

* Figures are based on data in Table 1.

Table 5 shows a disappearance of the "group effect" on oxygen consumption when the volume of water per fish is equal. Table 7, the corresponding table on locomotor activity, again contradicts the results of the table on oxygen consumption. Thus, it indicates that the smaller groups in the smaller volumes are the more active. However, if both Tables 5 and 7 are examined critically, it is found that the isolated fishes have a considerably higher rate of oxygen consumption and locomotor activity than do those in the group of two in twice the volume. On the other hand, the fishes in the group of two in both tables have a rate of oxygen consumption and locomotor activity that corresponds closely to that of the animals in the group of four in twice the volume. This again may mean that the isolated state is a unique one and is sufficient in these cases to overcome the handicap of a reduced total volume (the volume per fish being the same) in manifesting its effect on oxygen consumption and locomotor activity. Evidently, the relation of the volume of water per fish and the dimensions of the container to oxygen consumption and locomotor activity is a complex which requires further analysis.

TABLE 6

COMPARING THE ACTIVITY OF FISHES IN ISOLATION, GROUPS OF TWO, AND OF FOUR IN 6 LITERS WITH THE ACTIVITY OF SIMILAR COMBINATIONS IN 12 LITERS*

SIX LITERS		TWELVE LITERS	
Grouping	"Movements"† per Fish per 5-Minute Period of Observation	Grouping	"Movements"† per Fish per 5-Minute Period of Observation
Isolated	82.9 137.8 136.8	Isolated	76.7 154.0 163.5
Group of two	48.0 76.1 81.1	Group of two	48.9 76.7 78.3
Group of four	54.8 74.9 61.5	Group of four	45.4 65.7 73.9
Mean	83.8	Mean	87.0

Statistical significance of the difference, .4982

---

* Figures are based on data in Table 2.

† See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."

Schuett (1934), using goldfishes 3.5–4.0 cm. in length, found minimal locomotor activity in a 7.5-liter aquarium in a group of four and in a 15-liter aquarium in a group of eight. Thus, in the smaller aquarium the activity of a group of eight was higher than that of a group of four even though less water was available, per fish, to those in the larger group.

Escobar, Minahan, and Shaw (1936), working with goldfishes in isolation and in groups of four, found not a regular increase of activity with an increase in the volume per fish but a volume at which minimal activity occurred.

No minimal rates of oxygen consumption and locomotor activity were found in the present studies but might be discovered by using larger groups and larger aquaria.

TABLE 7

COMPARING THE ACTIVITY OF FISHES IN ISOLATION OR GROUPS OF TWO IN 6 LITERS WITH THAT OF FISHES IN GROUPS OF TWO OR FOUR IN 12 LITERS WHEN THE VOLUME OF WATER PER FISH IS EQUAL*

SIX LITERS		TWELVE LITERS	
Grouping	"Movements"† per Fish per 5-Minute Period of Observation	Grouping	"Movements"† per Fish per 5-Minute Period of Observation
Isolated.	82.9 137.8 136.8	Group of two. . .	48.9 76.7 78.3
Group of two. . . .	48.0 76.1 81.1	Group of four. . .	45.4 65.7 73.9
Mean.	93.8	Mean.	64.8

Statistical significance of the difference, .0424

* Figures are based on data in Table 2.

† See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."

"CONDITIONED" WATER

In an attempt to determine whether there was a chemical factor involved in the "group effect," the following procedure was used. The fishes were placed in isolation, groups of two and of four in aquariums in fresh lake water for a 21-hour period of acclimatization prior to the actual test. However, instead of being transferred to fresh lake water again for the tests, as was the procedure in the controls, they were kept in the same water that had been used for acclimatization. This water was called "conditioned" because fishes had lived in it and had added chemical substances to it. The animals used in these "conditioned"-water experiments were fed once a week, but at least 48 hours were allowed to elapse after feeding before any tests were run. According to Allee, Oesting, and Hoskins (1936), "the amount of conditioning is approximately a factor of the number and the size of fishes present, together with the volume of the water to which they were exposed, and may be indicated by a "conditioning coefficient."[2] In these experiments, on both oxygen consumption and locomotor activity in "conditioned" water, the coefficients were: 15 and 7.5 for an isolated fish in 6 and 12 liters, respectively; 30 and 15 for a group of two in 6 and 12 liters, respectively; and 60 and 30 for a group of four in 6 and 12 liters, respectively.

The controls were run with fishes placed into fresh lake water for the test period. The animals in the "conditioned"-water experiments were transferred to aquariums serving as temporary receptacles, while an oil film was placed on the "conditioned" water. They were then replaced into the test aquarium. This procedure assured an equal effect of handling in both controls and experimentals.

TABLE 8

COMPARING THE OXYGEN CONSUMPTION OF FISHES IN "CONDITIONED" WATER WITH THE OXYGEN CONSUMPTION IN UNCONDITIONED WATER*

"CONDITIONED" WATER		UNCONDITIONED WATER	
Grouping	Mean Cc. of O_2 Consumed per Fish	Grouping	Mean Cc. of O_2 Consumed per Fish
Isolated	6.50† 7.54	Isolated	7.85 8.76
Group of two	3.91 3.54	Group of two	5.82 7.02
Group of four	3.72 4.15	Group of four	5.07 6.18
Mean	4.89	Mean	6.78

Statistical significance of the difference, .0030

* Figures are based on data in Table 1.

† Each figure in the "conditioned" column represents the same volume of water as does each corresponding figure in the unconditioned column.

The "group effect" in "conditioned" water is listed under G and H in Tables 1 and 1*a* and under F and G in Tables 2 and 2*a*. The controls are E and F in Tables 1 and 1*a* and D and E in Tables 2 and 2*a*. In general, the results for "conditioned" water coincide with those for unconditioned water as far as the "group effect" is concerned. The isolated animal is found to be the most active and to have the highest rate of oxygen consumption. Tables 8 and 9 contain a direct comparison of oxygen consumption and locomotor activity of fishes in the same types of grouping in "conditioned" and unconditioned waters.

Table 8 indicates that fishes in "conditioned" water have a significantly lower rate of oxygen consumption than do those in unconditioned water. The explanation of this result is not clear at this time. Table 9 on locomotor activity shows no difference in activity between fishes in "conditioned" and unconditioned water. Again this may mean that, as used, the oxygen-consumption tests are the more sensitive.

---

[2] The conditioning coefficient is obtained by dividing the product of the number of conditioning fishes times their length in millimeters by the number of liters of water conditioned (Allee, Oesting, and Hoskins, 1936).

TABLE 9

COMPARING THE ACTIVITY OF FISHES IN "CONDITIONED" WATER WITH THE ACTIVITY IN UNCONDITIONED WATER*

"Conditioned" Water		Unconditioned Water	
Grouping	"Movements"† per Fish per 5-Minute Period of Observation	Grouping	"Movements"† per Fish per 5-Minute Period of Observation
Isolated..........	136.8‡ 163.5	Isolated.........	137.8 154.0
Group of two....	81.1 78.3	Group of two....	76.1 76.7
Group of four.....	61.5 73.9	Group of four. ..	74.9 65.7
Mean........	99.2	Mean......	97.5

Statistical significance of the difference, .6718

---

* Figures are based on data in Table 2.

† See discussion of locomotor activity under "Materials and Methods" for explanation of term "movement."

‡ Each figure in the "conditioned" column represents the same volume of water as does each corresponding figure in the unconditioned column.

TABLE 10

EFFECT OF NUMBERS PRESENT UPON THE AMOUNT OF OXYGEN CONSUMED BY GROUPED AND ISOLATED GOLDFISHES*

Isolated		Group of Four		Mean Difference	P-Value†
No. of Cases	Mean Cc. of O_2 Consumed per Fish	No. of Cases	Mean Cc. of O_2 Consumed per Fish		
40×1	4.60	10×4	3.22	1.38	.0009

* Test period is 3 hours. Volume is 6 liters of unconditioned water in a 7.5-liter aquarium.

† See Table 1a for discussion of *P*-value.

PARTIAL RECHECK OF RESULTS, USING MANOMETRIC METHOD

Since it was thought that even the modified method of Winkler, used in determining dissolved oxygen in the oxygen-consumption tests, might possibly contain some hidden error, it was decided to repeat some of the tests, using the Van Slyke manometric method (Van Slyke and Neill, 1924; Oesting, 1934). Allee and Oesting (1934) and Wilder (1937) tested the relative accuracy of the modified Winkler and Van Slyke methods and found agreement.

The materials and methods were the same as those described for the oxygen-consumption experiments using the modified Winkler method. Experimental conditions were identical with those of the earlier series. The oxygen consumption of an isolated fish was compared with that of one in a group of four. The results are summarized in Table 10. The statistical significance of the results is high and shows again that, other things being equal, the oxygen consumption of an isolated fish is higher than that of one in a group of four; this confirms results listed in Table 1. Thus, in so far as tested, findings obtained with the modified Winkler method are confirmed by the Van Slyke manometric method. This agrees with the experience of Wilder (1937).

DISCUSSION

The explanation of the effect of grouping on the respiration and locomotor activity of goldfishes is still hypothetical. Schuett (1933) dismissed the possibility of oxygen tension exerting any effect, at least until the asphyxia level is reached. Toryu (1927) reached the same conclusion, and our own experience is similar. Schuett (1933) also dismissed the possibility that the increased production of carbon dioxide by grouped fishes was the responsible factor.

Escobar, Minahan, and Shaw (1936) offer the suggestion that an isolated fish has no obstruction in its path of motion and can continue along this path undisturbed. On the other hand, a fish in a group must constantly make adjustments to the presence of its neighbors. Though isolated fishes, when active, usually swim in one unobstructed plane, their activity is less constant, and much of it is composed of a series of sudden spurts. Grouped fishes usually swim in several planes; but their activity is much more constant, since they are constantly subjected to stimuli from others in the group.

Vision as an important integrating factor for aggregations of fishes has been demonstrated by Bowen (1931). She reported that blinded catfishes of the species *Ameiurus melas*, which in contrast to *C. auratus* is a more definitely schooling animal, did not aggregate and that normal fishes did not respond in the dark. Bowen (1931) also found that responses to touch are of great importance and that the resulting stimulus may perhaps be the fundamental cause of close aggregations, with vision serving only as a means by which the fishes find one another. Bowen (1932) showed that catfishes in groups of four have a greater rate of oxygen consumption per fish than do those in isolation. This is what would be expected from a closely schooling fish when the individuals push actively toward the center of the group.

Vision may be acting as a factor inhibiting the activity of grouped goldfishes. If so, provided other factors are not exerting an important effect at the same time, blinded goldfishes and goldfishes kept in the dark should not manifest a "group effect." Experiments are now in progress in our laboratory to explore this possibility. It may be that sight reactions will account for all of the observed phenomena of "group effect" in the goldfish. However, it is possible that vibrations of tails of fishes set up pressure disturbances detected perhaps by lateral line organs, or that other sense organs are involved, either directly or indirectly. Planned experiments include an analysis of these factors.

Our results on oxygen consumption confirm those obtained by Schuett (1933) when there was an error in his technique, and contradict his later results (1934) when the error had been corrected. Schuett (1934) did find a higher rate of locomotor activity for goldfishes in isolation than for those in groups of four. This result does not correlate with his failure to find a higher rate of oxygen consumption in the isolated fish, using the modified Winkler technique. Schuett's experiments on locomotor activity were performed in 7.5- and 15-liter aquariums as were our experiments. His experiments on oxygen consumption were performed in Erlenmeyer flasks; and Schuett offers a possible explanation of contradictory results, stating:

> This may be because of the fact that the respiratory chambers used in oxygen consumption work are of such a size as to produce a crowding effect upon the contained fishes, causing movements that would not otherwise occur. In such experiments the fishes were seldom quiet in the respiratory chambers, usually moving in vertical paths from bottom to top in the chambers. This was true both with isolated fishes and with groups of four. Under these circumstances the effect of quieting, as usually noticed in the groups, was lost as the results here indicate.

Our results confirm those obtained by Schuett (1934) and by Escobar, Minahan, and Shaw (1936) on the locomotor activity of isolated goldfishes and goldfishes in groups of four; and, volume comparisons excepted, a correlation has been established between oxygen consumption and locomotor activity.

It is appropriate here to compare the relative sensitivities of the Winkler chemical analysis used in determining the oxygen consumption and the observational technique used in determining the locomotor activity. In favor of the chemical technique it may be said that it detects two cases, B and C in Table 1, in which the oxygen consumption of the fishes in the group of two is significantly higher than that of the fishes in the group of four though, in general, the oxygen consumption of the two groups tends to be the same. The locomotor activity technique detects no difference in a similar comparison. Table 4 shows a significant increase in oxygen consumption of goldfishes placed in larger volumes, while Table 6 shows no corresponding effect on locomotor activity. Table 8 indicates a decreased oxygen consumption of fishes in "conditioned" water, while Table 9 shows no corresponding effect on the locomotor activity. In favor of the observational technique it may be said that it indicates more effectively in Table 7, comparing the isolated fish in 6 liters with the fishes in the group of two in 12 liters, that the isolated state may be sufficient to overcome the handicap of reduced total volume and to cause greater activity in the same volume of water per fish than does a similar comparison in Table 5 on oxygen consumption. Also, after Schuett (1934) had failed to obtain a "group effect" on oxygen consumption, using the modified chemical technique, he did get a "group effect" on locomotor activity, as did Escobar, Minahan, and Shaw (1936). However, the use by Schuett (1934) of Erlenmeyer flasks instead of aquariums for the tests on oxygen consumption must be borne in mind as a possible explanation. All things considered, it is the opinion of the writer that the chemical technique is the more sensitive one.

It is too early to attempt to discuss the broader implications of these and other studies on the mass physiology of the goldfish. In addition to the problems here attacked, other workers in this laboratory have found (*a*) that when exposed to toxic amounts of colloidal silver, grouped goldfishes survive longer than those in isolation (Allee and Bowen, 1932); (*b*) that in an appropriate, simple setup, grouped goldfishes learn more rapidly than isolated animals (Welty, 1934); (*c*) that goldfishes consume more *Daphnia* per unit of time if grouped than if isolated (Welty, 1934); and (*d*) that under many experimental conditions small, isolated goldfishes grow more rapidly in water in which other goldfishes have lived than in uncontaminated water (Allee, Bowen, Welty, and Oesting, 1934; Allee, Oesting, and Hoskins, 1936; Livengood, 1937). In time, perhaps these and other studies may permit a critical examination of the general survival-value of numbers of goldfishes. For the present it is useless to do more than to recognize that such general problems exist and that they are not dissociated from the present studies.

SUMMARY

1. In a given volume of water an isolated goldfish consumes more oxygen and has a higher rate of locomotor activity than does each fish in a group of two.
2. In a given volume of water an isolated goldfish consumes more oxygen and has a higher rate of locomotor activity than does each fish in a group of four.
3. In a given volume of water each goldfish in a group of two tends to consume the same amount of oxygen and to have the same rate of locomotor activity as does each fish in a group of four.
4. Fishes in "conditioned" water exhibit the same type of "group effect" as do those in unconditioned water.
5. Fishes in "conditioned" water consume less oxygen than do those in unconditioned water.
6. Volume relationships are complex and require further analysis.
7. The modified Winkler technique appears to be more sensitive in determining oxygen consumption than the observational technique in determining locomotor activity.
8. A partial recheck of oxygen-consumption experiments, using manometric methods, confirms the relationships summarized in conclusion No. 2.

LITERATURE CITED

ALLEE, W. C. 1927. Studies in animal aggregations: some physiological effects of aggregations in the brittle starfish, *Ophioderma brevispina*. Jour. Exper. Zool., 48:475–95.

———. 1931. Animal aggregations. Chicago: University of Chicago Press.

———. 1934. Recent studies in mass physiology. Biol. Rev., 9:1–48.

ALLEE, W. C., and BOWEN, E. S. 1932. Studies on animal aggregation: mass protection against colloidal silver among goldfishes. Jour. Exper. Zoöl., 61:185–207.

ALLEE, W. C.; BOWEN, E. S.; WELTY, J. C.; and OESTING, R. 1934. The effect of homotypic conditioning of water on the growth of fishes and chemical studies of the factors involved. Jour. Exper. Zool., 68:183–213.

ALLEE, W. C., and OESTING, R. 1934. A critical examination of Winkler's method for determining dissolved oxygen in respiration studies with aquatic animals. Physiol. Zoöl., 7:509–541.

ALLEE, W. C.; OESTING, R.; and HOSKINS, W. H. 1936. Is food the effective growth-promoting factor in homotypically conditioned water? Physiol. Zoöl., 9:409–32.

BOWEN, E. S. 1931. The role of the sense organs in aggregations of *Ameiurus melas*. Ecological Mono., 1:3–35.

———. 1932. Further studies of the aggregating behavior of *Ameiurus melas*. Biol. Bull., 63:258–70.

ESCOBAR, R. A.; MINAHAN, R. P.; and SHAW, R. J. 1936. Motility factors in mass physiology: locomotor activity of fishes under conditions of isolation, homotypic grouping, and heterotypic grouping. Physiol. Zoöl., 9:66–78.

HYMAN, L. H. 1929. The effects of oxygen tension on oxygen consumption in *Planaria* and some echinoderms. Physiol. Zoöl., 2:505–34.

KEYS, ANCEL B. 1930. Influence of varying oxygen tension upon the rate of oxygen consumption of fishes. Bull. Scripps Inst. Oceanog., Tech. Ser., 2:307–17.

LIVENGOOD, W. F. 1937. An experimental analysis of certain factors affecting growth of goldfishes in homotypically conditioned water. Copeia, No. 2, pp. 81–88.

OESTING, R. 1934. A modified Van Slyke method for the determination of dissolved oxygen and total carbon dioxide in water. Physiol. Zoöl., 7:542–49.

SCHUETT, J. F. 1933. Studies in mass physiology: the effect of numbers upon the oxygen consumption of fishes. Ecology, 14:106–22.

———. 1934. Studies in mass physiology: the activity of goldfish under different conditions of aggregation. *Ibid.*, 15:258–62.

"STUDENT." 1925. New tables for testing the significance of observations. Metron, 5:105–8.

TORYU, Y. 1927. The respiratory exchange in *Carassius auratus* and the exchange of the air bladder. Sci. Repts., Tohoku Imp. Univ., Ser. 3, 4:87–96.

VAN SLYKE, D., and NEILL, J. 1924. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. Jour. Biol. Chem., 61:523–73.

WELTY, J. C. 1934. Experiments in group behavior of fishes. Physiol. Zoöl., 7:85–128.

WILDER, J. F. 1937. A correlation of results on oxygen consumption obtained by the Winkler method and by respirometers, using as a standard the methods of Van Slyke. Physiol. Zoöl., 10:464–72.

FETAL RESPIRATORY MOVEMENTS IN THE CAT

(Two figures)

W. F. WINDLE,[1] M. MONNIER,[2] AND A. G. STEELE
Anatomical Laboratory and Institute of Neurology, Northwestern
University Medical School

FETAL respiratory movements have been described in several mammals, including the rat (Corey, 1932), rabbit (Snyder and Rosenfeld, 1937), sheep (Barcroft and Barron, 1936 and 1937), and man (Ahlfeld, 1905; Windle, Dragstedt, Murray, and Green, 1938). The present study in the cat was undertaken to compare the movements of respiration at various stages in fetal life and under conditions as nearly normal as possible, as well as in states of experimental fetal anoxemia and carbon dioxide excess. We wished to learn whether or not the fetus responds to conditions which serve as respiratory stimuli in the adult.

Cats in various stages of pregnancy were anesthetized with ether before ligating the carotid arteries high in the neck and the basilar artery at the rostral one-third of the pons (Pollock and Davis, 1924) an hour or more (usually 2 or 3 hours) before experiments were begun. With this procedure the animals entered a state of partial decerebration. Conscious voluntary activity was lost; but respiration, although temporarily impaired by the procedure, was usually restored within an hour or two to about normal and, in several instances, was considerably increased in rate and depth; body temperature and blood pressure were usually not affected significantly. The explanation for the maintenance of rapid breathing and normal temperature seems to be that enough blood reached the midbrain and diencephalon by collaterial circulation around the ligature on the basilar artery to support the integrity of centers there even though the cerebral hemispheres were quite lacking in blood flow. After the vessels had been ligated, ether was discontinued; later on, the cat's abdomen could be opened under physiological saline or Locke's solution in a constant-temperature bath with no further anesthesia. All the fetuses exposed were delivered through small openings in the uterus at the ends of the zonary placentas and were examined with placental circulation intact. Various experimental procedures were used during the course of the study of fetal reactions.

RESULTS IN SPECIMENS OF LESS THAN 40 DAYS' GESTATION

In a series of fifty embryos and small fetuses 13.5–30 mm. length (not listed in Table 1), we observed no rhythmical movements of respiration. However, in the largest (those of 25–30 mm.), occasional and nonrhythmical contractions of diaphragm, abdominal, and intercostal muscles occurred. Some time after exposing the specimens, these movements began to take place with simultaneous extension of head, trunk, and extremities. These integrated activities seemed to be the precursors of truly rhythmical respiration but were brought on by impairing the fetal oxygen-exchange mechanism in delivering the specimens.

---

[1] Aided by a grant from the American Academy of Arts and Sciences.

[2] Fellow of the Rockefeller Foundation.

Rhythmical respiratory movements were encountered in all the 122 cat fetuses listed in Table 1, but the nature of the movements varied with the age and size of the specimens and with the physiological conditions obtaining at the time of the experiments. Fetuses younger than about 40 days of gestation (32–58 mm. C. R. length) could usually be exposed in the constant-temperature bath and observed for a few minutes before we noticed the marked changes in the blood color which would signify anoxemia. In none of these specimens viewed under the best of conditions did we observe rhythmical respiratory movements. The specimens often showed movements of the head, limbs, trunk, or tail; but these activities appeared to be random and nonintegrated; the fetuses were definitely apneic in respect to respiratory movements. Invariably they began to show respiratory movements a few minutes after delivery, and these soon became rhythmical. This change in behavior appeared to be correlated with constriction of the uterine vessels as the uterine muscle tightened upon the placenta. When left undisturbed in the bath, fetal respiration increased in rate and depth but seldom became constant. Instead, it was intermittent, and the periods of breathing movements seemed to correspond to times when the fetal blood color was somewhat darkened.

TABLE 1

EXPERIMENTS IN WHICH RHYTHMICAL RESPIRATORY MOVEMENTS OF CAT FETUSES WERE STUDIED

Measurements are crown-rump length of fresh specimens. Age is in time after observed matings, except where marked with an asterisk (*), which indicates computation from an age-length curve.

CAT NO.	LIVING FETUSES	FETAL SIZE		FETAL AGE (DAYS)	CAT NO.	LIVING FETUSES	FETAL SIZE		FETAL AGE (DAYS)
		Length (Mm.)	Weight (Gm.)				Length (Mm.)	Weight (Gm.)	
1	3	32	....	33	17	3	80	....	44
2	6	32–35	....	32	18	6	80	....	?
3	4	35	2.6–3.0	32–33*	19	5	85–90	28–33	?
4	4	35	....	32–33*	20	4	85–90	....	?
5	4	38–40	....	33–34*	21	4	95	36–42	?
6	3	38–40	....	33–34*	22	3	91–101	14–17	?
7	4	40	....	34*	23	1	100	....	51
8	3	48–50	....	?	24	2	100	....	?
9	5	45–52	....	?	25	5	100–104	38–46	?
10	5	54–56	....	39	26	5	110–15	....	53
11	4	55–58	....	?	27	4	115	70–76	?
12	4	65–70	....	44	28	3	120	....	?
13	4	70–72	....	?	29	3	135	....	62
14	4	60–75	....	?	30	3	140	....	63
15	4	75	18–19	?	31	4	140	....	65
16	2	75–80	15–20	?	32	4	140–46	107–27	?

The first rhythmical respiratory movements to manifest themselves upon exposure of the fetuses were usually quite delicate, quick, unsustained contractions of the diaphragm, intercostal, and abdominal muscles, and, in a few instances apparently, of the diaphragm alone. With prolonged exposure, more and more muscles shared in this reaction until each "breath" appeared to be a gasp involving extension of the head and limbs. These violent gasplike respirations never occurred in fetuses whose umbilical veins were of good color. Even when the exposed fetuses were executing marked rhythmical respiratory movements, those *in utero* showed no such activity but could often be seen to move the feet, tail, or head arhythmically.

It was impossible to avoid the implication that fetuses less than 40 days old (58 mm.) were apneic in decerebrated cats and that respiration came about with a state of partial anoxemia which could not be avoided in exposing the fetuses experimentally. To test this further, we clamped the umbilical cords of fetuses immediately after exposing them. Half a minute or more usually elapsed before results appeared. Some of the fetuses executed a few rapid and rhythmical contractions of the diaphragm, usually with concomitant action of intercostal and abdominal muscles; other quick, jerklike movements were often seen. Within a few seconds to half a minute lively squirming (tonic mass convulsive movements) and running movements were observed. Irritability to mechanical stimulation then began to decrease, and muscle tonus increased; the fetus slowly assumed a posture resembling decerebrate rigidity, owing to sustained contraction of trunk and limb muscles. This sustained contraction was interrupted by rhythmical gasps which had begun during the phase of active squirming. Gasping was the last motor phenomenon to stop as death approached. At first the head extended with each gasp; but as depression came on, this changed to flexion. Even after all irritability was lost and when the last feeble gasp had occurred, re-establishment of the placental circulation sometimes led to partial recovery. Occasionally the motor responses were imperfectly re-enacted during recovery, but seemingly in the reverse order of their manifestation during the asphyxia. The phenomena of asphyxia could usually be repeated by clamping the umbilical cord again. Fetal apnea was not permanently resumed after asphyxia; the fetuses usually showed interrupted respiratory rhythms, which seldom involved the delicate movements of the primary breathing muscles alone, until the end of the experiment.

Administration to the mother cats of mixtures of 5–8 per cent oxygen in nitrogen by gas machine and tracheal canula brought about cyanosis, indicated by darkening of the uterine vessels, and respirations of previously apneic fetuses started promptly. It was evident that the changes in behavior observed under asphyxia induced by oxygen lack resembled, but in exaggerated form, those seen when the fetuses were exposed in the bath for prolonged periods; and we concluded that the delivery of the fetus had, indeed, set up a state of partial asphyxia which brought about fetal respirations.

We tested the effects upon fetuses of carbon dioxide given to the mother cats in mixtures of 5–20 per cent in oxygen. With the animals under the influence of 8–10 per cent carbon dioxide the fetuses which had been apneic or showed only an occasional arhythmical respiratory movement when handled at delivery began to execute lively rhythmical respiratory movements, as is illustrated in Figure 1. In other fetuses of the same litters, but which had been exposed for some time, the existing respiratory movements became more rapid and the depths of each increased. Furthermore, even in the smallest so tested (35 mm.), the unexposed fetuses *in utero*, in which we could neither palpate nor see respiratory movements before giving the gas, began to execute rhythmical respirations when the cat breathed the oxygen–carbon dioxide mixture. The color of the umbilical veins and of the fetuses themselves was very good before and during the time the mixtures were given, and there was no visible indication of asphyxia. When the mother cats whose fetuses were apneic were overventilated with pure oxygen there was no observable stimulation of respiratory activities in the fetuses. We concluded that the carbon dioxide had acted in its usual capacity to stimulate respiration.

Prolonged breathing of high concentrations of carbon dioxide (15–20 per cent) in oxygen led to fetal gasping and depression of the delicate rapid respiratory rhythm; but when air or oxygen was substituted, a rebound stimulation of the respiratory rate like that seen in bird fetuses (Windle and Nelson, 1938) sometimes ensued. In a few experiments the cat fetuses resumed their apneic state for brief intervals after the effects of carbon dioxide had subsided.

RESULTS IN SPECIMENS OF 62–65 DAYS' GESTATION

Gestation in several cats which were mated in our laboratory lasted 64 days. In the hope that new corpora lutea would inhibit uterine contractions (Snyder and Rosenfeld, 1937), attempts were made to bring about ovulation in two cats by administration of pregnancy urine concentrate (Parke Davis' Antuitrin S.) 6 days before the end of gestation. One cat (No. 31) ovulated, apparently on the sixty-fourth day; and the fetuses were studied the following day. Results in this animal were similar to those in the other three cats and will be described with them. The uterus was quite flaccid for the first half-hour.

The observations of fetal respiratory movements in the fourteen fetuses of the cats of 62–65 days' gestation (fetuses 135–146 mm. long) differed significantly from those in the fetuses less than 40 days old. In the first place, after the effects of ether used during the tying of carotid and basilar arteries had subsided, intrauterine activities of a rhythmical nature could be palpated through the intact maternal abdomen in two of the animals. These were very inconstant, occurring at long intervals as short series of rapid beats. They suggested to the observers similar phenomena encountered after opening the abdomen and which were unquestionably respiratory movements.

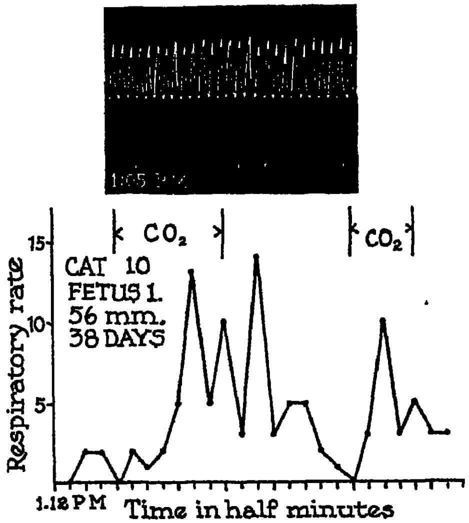


FIG. 1.—The kymograph record illustrates maternal respiration of the cat whose carotid and basilar arteries were ligated at 10:30 A.M., taken just before the animal was placed in the bath and abdomen opened, time in 5-second intervals The graph illustrates production of fetal respiration at 38 days' gestation by administering 8–10 per cent carbon dioxide in oxygen to the mother cat through tracheal canula. The lag is caused in part by the time it took to get the gas through the delivery tube of the machine; this factor was later controlled by compressing the bags just before administering the gas (see Fig. 2).

Intrauterine respiratory movements were seen in all four animals after opening the abdomen, and were likewise of short duration and occurred at intervals. Upon exposing the fetuses through openings in the uterus made at ends of the zonary placentas, the umbilical-vein blood was found to be very dark and the fetuses seemed to be quite depressed. After the specimens had been exposed with placental circulation intact for several minutes, they usually began to execute rhythmical respiratory movements, which varied in rate from about 10 to 30 or more per minute and occurred intermittently. The intermittent occurrence of the fetal respiratory rhythm appeared to be associated with contraction of uterine musculature and accompanying improvement in fetal blood color. When a fetus was delivered in the relaxed phase of the uterus, its umbilical vessels were dark and it was apneic. Contraction of the uterus brought about a reddening of the blood in the umbilical veins, and the fetal color improved. As the contraction was subsiding, fetal respiratory activity began and persisted until the umbilical veins darkened again. This series of events occurred in slow rhythm of less than once to as many as four times a minute. When a fetus was delivered from the uterus during a strong uterine contraction, its umbilical veins were somewhat redder and it was executing rhythmical respiratory movements by the time we could get it out. The better the color of the umbilical veins, the more rapid was the fetal respiratory rate and the fewer the muscles involved in each "inspiration." However, delicate respiratory movements, such as were seen in young fetuses, were never encountered in those near term; all involved more muscles than the diaphragm alone.

In none of the cats of 62–65 days' gestation were we able to increase the rate of intrauterine fetal respirations by administering oxygen–carbon dioxide mixtures to the mother cat, nor were we able to start fetal respirations in the apneic exposed fetuses except by bringing about uterine contractions and reddening of fetal blood. On the contrary, mixtures of 5–15 per cent carbon dioxide and oxygen served only to deepen the inspirations, after which they stopped entirely. When oxygen alone was forced upon the cat, respiratory movements of the fetus sometimes began or the rate increased.

These experiments seem to show that fetuses near term, quite unlike those earlier described, were actually somewhat depressed and in a state of partial anoxemia. Conditions which prejudiced their placental exchange mechanism still more did not bring about rapid rhythms of respiratory movements. Conditions which tended to relieve the anoxemia did bring about breathing rhythms. The rhythmical contraction of the uterus itself was a factor acting to relieve the anoxemia intermittently, at least in those specimens which had been delivered with placentas still intact.

RESULTS IN SPECIMENS OF 40–60 DAYS' GESTATION

As might be expected from a consideration of the remarkable differences in results obtained in young and old fetuses of the cat, the observations in specimens of intermediate age were subject to considerable variation. Taken alone, these would prove to be most confusing. With physiological conditions better in some animals than in others, it was to be expected that we would find differences in the degree of oxygenation of the various litters of fetuses. Judging from fetal blood color, this is essentially what we observed. Furthermore, removal of a large fetus from a greatly distended uterus is followed by alterations in the placental exchange mechanism which are much more pronounced than after removal of a smaller fetus, the placentas in the two instances being much the same size. Since we cannot summarize all the experiments of this group in a single description, we propose to illustrate the principal variations.

In cat No. 17, whose fetuses measured 80 mm. in length, conditions appeared to be as good as could be expected. The basilar and carotid arteries had been tied under ether between 9:45 and 10:15 A.M., after which the anesthetic was discontinued; the animal was kept at rest until 1:15 P.M., when the abdomen was opened in the constant-temperature bath. The cat developed some decerebrate rigidity, but there was no depression of its respiration, which, on the contrary, was rapid and deep. Its temperature was unaffected. During the first 8 minutes after delivering the first fetus there were no rhythmical fetal respiratory movements (Fig. 2); the cat was breathing pure oxygen much of the time. Four respirations occurred without distinct rhythm in the first half-minute after exposing the fetus, and were probably due to handling the fetus; otherwise, only an occasional slight, local movement of the diaphragm with elevation of the costal margin was seen. The undelivered fetuses *in utero* were quite apneic. Administration of 8–10 per cent carbon dioxide in oxygen to the mother cat brought about marked respiratory acceleration in the mother cat and simultaneously respiratory movements began in all the fetuses. The respirations of the delivered fetus quickly reached a maximum of 32 in half a minute, then became very deep, involving all respiratory muscles, and began to subside as though the gas were exercising a secondary depressing effect. With oxygen alone following this, fetal respirations again improved suggesting that a certain amount of rebound stimulation occurred as the carbon dioxide excess was being removed. In this experiment the results obtained were like those described in the small fetuses and differed from those observed in most of those 80–120 mm. in length, whose respiratory behavior was much more like that of the larger specimens near term.

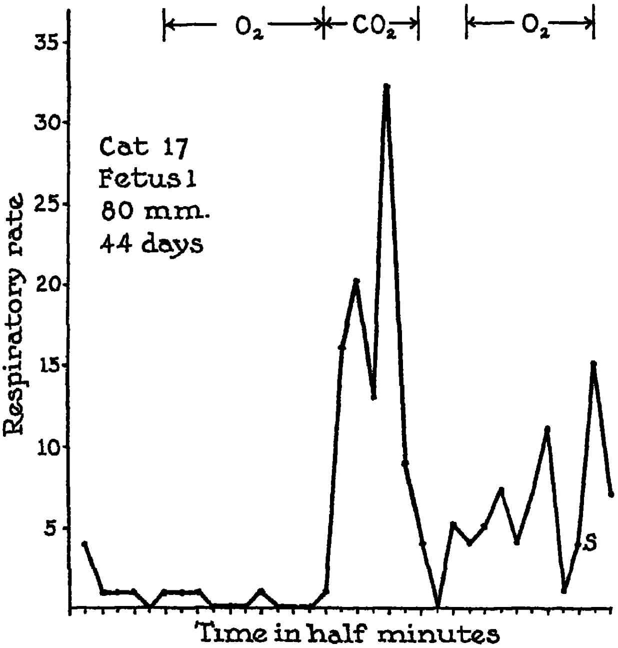


FIG. 2.—Illustrates production of fetal respiration at 44 days by 8–10 per cent carbon dioxide in oxygen. The percentage of carbon dioxide delivered by the machine was controlled for all experiments by sampling and analyzing by the Van Slyke method.

It was usually found that 80–120 mm. fetuses executed rhythmical respiratory movements after delivery with placentas intact. These occurred at the conclusion of each rhythmical uterine contraction and showed improvement when the cats were over-ventilated with oxygen. In some experiments carbon dioxide–oxygen mixtures served to increase rate, as well as depth, of fetal respirations; but it was seldom entirely clear that the increase in rate was not due to an increased oxygenation of the fetuses accompanying augmentation of uterine activity. The first delivered 100-mm. fetus of cat No. 23 illustrates the point in question. The fetus was quite anoxemic when first exposed, and rhythmical respiratory movements were not stimulated when the mother cat was allowed to rebreathe the air in the tube to the gas machine for 4 minutes. But administration of a mixture of 5–8 per cent carbon dioxide in oxygen 6½ minutes after exposing the fetus led to rhythms of fetal respiratory movements reaching 52 per minute. The condition of the mother cat of this experiment seemed to be satisfactory. Perhaps the removal of so large a fetus from a distended uterus had led very suddenly to a condition of anoxemia in the fetus. The oxygen–carbon dioxide mixture apparently stimulated uterine activity, which helped overcome the anoxemia by delivering oxygenated blood to the fetus intermittently.

When experiments were impaired by poor maternal respirations, by increased body temperature, or by decreased maternal blood pressure after ligating the carotid and basilar arteries, the results obtained in the fetuses, even in earlier stages of pregnancy, resembled those recorded under good conditions in fetuses near term. In cat No. 8 (fetuses 48–50 mm. long) experimental conditions after tying carotid and basilar arteries were not as good as usual, and the maternal respiration had to be strengthened by allowing the cat to breathe the oxygen–carbon dioxide mixture for about 5 minutes before we opened the abdomen. The fetuses showed no respiratory movements of the rhythmical type, but they did gasp occasionally. The specimens seemed to be depressed to the point that they could not be stimulated by the carbon dioxide breathed by the mother. When the cat was forced to breathe oxygen alone, rhythmical respiratory movements of the fetus began, reaching a maximum of 34 per minute. It would be hazardous to conclude that results of this nature were representative of normal activities *in utero*. All the experimental conditions must be carefully evaluated.

DISCUSSION

The experiments in cat fetuses of various ages throw some light upon the subject of prenatal respiratory activity and also bring up several questions. We should like to know something more definite about the relationship between fetal and placental blood oxygen and carbon dioxide and fetal respiratory movements. What concentrations of these gases are compatible with the phenomena in question? Such studies have been started. The relationship between uterine contraction and the intermittent occurrence of prenatal respiratory rhythms must be investigated further before we can complete the picture of fetal activities *in utero*. Furthermore, the present studies have demonstrated the importance of correlating observations on the physiological conditions of the maternal animals with the responses obtained in the fetuses. This must receive even greater attention in the future.

There can be little doubt that cat fetuses under normal physiologic conditions execute rhythmical respiratory movements *in utero* in the later days of gestation. Our own observations add evidence to that presented by Ahlfeld (1905) and by Snyder and Rosenfeld (1937). We believe that this activity is manifested in the cat because the fetuses are experiencing a physiologic anoxemia *in utero*. How extensive such an anoxemia is cannot be told until analyses of the fetal blood have been made under satisfactory experimental conditions. If we may judge at all by color of the umbilical veins and the fetuses themselves, it would appear to be rather marked.

If the cat fetuses near term were already profoundly anoxemic *in utero*, it could not be expected that they would react to conditions of asphyxia or carbon dioxide excess with an increase in respiratory rate. On the contrary, such conditions should lead to further depression. This is what our experiments demonstrated. It is what Snyder and Rosenfeld (1937) found in rabbits at term and in those whose gestation had been prolonged up to 35 days by causing new corpora lutea to be developed before the end of the period and thus inhibiting uterine contractions. It may be significant that Koff and Davis (1937) have demonstrated in rabbits whose labor was inhibited by such methods that fetuses did not live beyond the thirty-sixth day. Perhaps they died of asphyxia. We observed that, during complete flaccidity of the cat's uterus, the fetuses were quite depressed and their blood color was dark. But when contractions of the uterus took place, color improved and the specimens were aroused to the point of executing rhythmical respiratory movements. Are the prelabor contractions of the uterus physiological, and do they serve the very useful purpose of preventing asphyxia in the fetus?

Our experiments in the younger cat fetuses (40 days and less) demonstrated a condition quite different from that obtaining near term. These small fetuses were apneic under normal physiological conditions, but rapid and delicate fetal respiratory movements of a rhythmical nature could be initiated by administering carbon dioxide to the mother or by causing asphyxia of the fetuses. It is reasonable to suggest that observers who have been unable to demonstrate such a stimulating or augmenting action of carbon dioxide have been dealing with specimens already stimulated in a state of anoxemia.

It is impossible for us to say how late in the gestation period fetuses normally are apneic *in utero*. Perhaps as late as the forty-fourth day (80 mm.) or longer. It must depend upon the time in the prenatal life when a physiologic state of anoxemia begins to manifest itself, and this may be an intermittent phenomenon subject to variation.

Observations were obtained in the chick (Windle and Barcroft, 1938; Windle, Scharpenberg, and Steele, 1938) and duck (Windle and Nelson, 1938) which were similar to those in the cat. It was found that carbon dioxide stimulated fetal respiration during the greater part of the life in the egg and that only when normally occurring respirations had attained a rapid rate at the time the respiratory allantois ceased to function efficiently did it begin to act as a depressing factor.

Although we have attempted to gain an understanding of the intrauterine respiratory movements obtaining normally, we have not considered the relationship of the nervous system to these phenomena in the cat fetuses in the present investigation. As Barcroft and Barron (1937) have demonstrated for the sheep, the fetuses' own higher nervous centers seem to play important parts in inhibiting fetal activity including respiration. It is quite possible that such factors come into play at relatively different times in the cat and sheep. In the cat fetuses of 32 days' or more gestation which appeared to be apneic *in utero* and when first delivered, inhibitory influences of the brain may already be at work. We know that descending tracts from the tegmentum and mesencephalic tectum are well formed much before the middle of fetal life (Windle, 1935). This subject will receive more consideration at a later time. In respect to humoral factors influencing respiratory activities, it is evident, from the present studies, that cat fetuses react very much as do adult animals to the usual respiratory stimuli.

CONCLUSIONS

Cat fetuses tended to be apneic *in utero* during the middle third or more of the gestation period, but factors leading to asphyxia served to inaugurate delicate rapid rhythms of respiration movements.

Severe and prolonged asphyxia depressed the delicate movements, but gasping occurred and persisted after finer movements and irritability to mechanical stimulation were lost.

Carbon dioxide breathed by the mother cat started rhythmical respiratory movements of fetuses normally apneic, but served only to depress further those fetuses which were in a state of partial anoxemia.

Near term, fetuses 62–65 days old executed breathing movements normally *in utero*. Anoxemia and carbon dioxide in excess did not stimulate, but depressed, the respiratory activities of these specimens.

Normally occurring but intermittent rhythms of intrauterine respiratory movements were co-ordinated with phasic uterine contractions. They seemed to result from improved oxygenation of the fetuses at the time of increased uterine tone.

LITERATURE CITED

AHLFELD, F. 1905. Die intrauterine Tätigkeit der Thorax und Zuerchfell Muskulatur. Intrauterine Atmung. Monatschr. f. Geburtsch. u. Gynäk., 21: 143–63.

BARCROFT, J., and BARRON, D. H. 1936. The genesis of respiratory movements in the fetal sheep. Jour. Physiol., 88: 56–61.

———. 1937. Movements in midfoetal life in the sheep embryo. *Ibid.*, 91: 329–51.

COREY, E. L. 1932. Initial inspiration in the mammalian fetus. Jour. Exper. Zoöl., 61: 1–11.

KOFF, A. K., and DAVIS, E. M. 1937. The mechanism of the prolongation of pregnancy in the rabbit. Amer. Jour. Obst. and Gynec., 34: 26–37.

POLLOCK, L. J., and DAVIS, L. E. 1924. Studies in decerebration. I. A method of decerebration. Arch. Neurol. and Psychiat., 10: 391–98.

SNYDER, F. F., and ROSENFELD, M. 1937. Direct observation of intrauterine respiratory movements of the fetus and the role of carbon dioxide and oxygen in their regulation. Amer. Jour. Physiol., 119: 153–66.

WINDLE, W. F. 1935. Neurofibrillar development of cat embryos: extent of development in the telencephalon and diencephalon up to 15 mm. Jour. Comp. Neurol., 63: 139–71.

WINDLE, W. F., and BARCROFT, J. 1938. Some factors governing the initiation of respiration in the chick. Amer. Jour. Physiol., 121: 684–91.

WINDLE, W. F.; DRAGSTEDT, C. A.; MURRAY, D. E.; and GREEN, R. R. 1938. A note on the respiration-like movements of human fetuses. Surg., Gynec., and Obst., 66: 987–88.

WINDLE, W. F., and NELSON, D. 1938. Development of respiration in the duck. Amer. Jour. Physiol., 121: 700–707.

WINDLE, W. F.; SCHARPENBERG, L. G.; and STEELE, A. G. 1938. Influence of carbon dioxide and anoxemia upon respiration in the chick at hatching. Amer. Jour. Physiol., 121: 692–99.

PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER. II. GENERAL PRINCIPLES OF DEVELOPMENT WITH SPECIAL REFERENCE TO THE AFTER-FEATHER

(Eighteen figures)

FRANK R. LILLIE AND MARY JUHN
Whitman Laboratory, University of Chicago

CONTENTS

I. Introduction 434
II. Growth and movement in the feather germ 435
  A. Axial growth 435
  B. Radial growth 436
  C. Origin and growth of the barbs and their tangential movement 436
    1. Axial growth of the germ in relation to the barbs 437
    2. Tangential growth in relation to the barbs 437
    3. Barbs of irregular length 437
    4. The cellular basis of origin and growth of the barbs 439
    5. The origin of barb apexes 439
    6. Analysis of tangential movement 440
  D. The formation of the shaft and the role of concrescence 441
  E. Summary 442
III. The after-feather 443
  A. Introduction 443
  B. Development 444
    1. Irregularities in the ventral locus 444
    2. The origin of the first afterbarbs 445
    3. Origin of bilateral symmetry of the after-feather 445
    4. Later development of the after-feather 445
    5. Discussion 446
  C. Patterns in the definitive after-feather and the main feather 447
Literature 448

I. INTRODUCTION

FEATHERS represent a special kind of biological system beautifully adapted on account of the relative simplicity and symmetry of structure and pattern to a great variety of biological problems. Their special suitability as test objects for hormones and various physiological conditions and for genetical problems, especially on the borderline between genetics and physiology, is being very generally recognized. Basic to the interpretation of whatever biological problem for which they may furnish material is an understanding of their development. The present paper is, in a sense, a continuation of our interrupted first study in this field published in 1932. The collaborative studies of Juhn and Fraps (1934, 1936), the criticism of Hosker (1936), and our own experience since have resulted in some clarification and modification of views expressed in 1932, as well as additional material, and it seems necessary, therefore, to state these, though only rather categorically in the present paper, before proceeding to the special consideration of the after-feather. As a matter of fact, a correct understanding of the special problem depends on the more general ones.

The present study is not comparative as between species, although we explicitly recognize the importance of such studies; but we believe that they will proceed more successfully on the basis of a fairly complete analysis in a single form. The study of the regeneration of certain definitive feathers in two breeds of fowl and crosses between them is, in fact, a large undertaking. Our study is limited to them, and in the introductory considerations, to a partial presentation of certain general problems.

II. GROWTH AND MOVEMENT IN THE FEATHER GERM

The feather germ grows in length, *axial growth;* its base (collar) grows somewhat in diameter, *radial growth;* and the barbs, arising in the "ventral" region of the collar, progress in their growth around the periphery of the collar from ventral to dorsal position, *tangential growth or movement.* The foregoing dimensions of growth have reference to the germ as a whole. The barbs and subordinate elements also present their special problems of growth.

A. AXIAL GROWTH

The growing feather presents an apparently straight axis, so long as it is entirely inclosed in its sheath, when it is commonly called the pinfeather. The gentle dorsoventral curvature characteristic of certain contour feathers is not then apparent. From this we conclude that axial growth from the collar is uniform at all transverse levels, or at least very nearly so. Certain important conclusions emerge from this fact, among them the principle that transverse sections parallel to the collar, which is the center of axial growth, intercept points of identical or nearly identical age from the dorsal to the ventral surface.

On the other hand, and without any prejudice to the principle of equal axial growth at all transverse levels, the rate of axial growth is subject to variation in time which has been measured and recorded in the form of curves by Juhn, Faulkner, and Gustavson during formation of the individual feather and in different feather tracts (1931).

The collar is the source of the axial growth, and it is necessary, therefore, to analyze its activities in order to appreciate how the axial growth is initiated and sustained. Figure 1 is a photograph of a frontal (longitudinal) section of the base of a feather germ of 18 days' regeneration from the breast of a Brown Leghorn capon. It shows the regenerating feather in its follicle. Attention is directed at this time to the thick obliquely placed collar surrounding the umbilicus where the central artery enters, hence appearing on both sides, and to the sharp angle that separates it from the forming barbs. In the higher magnification of the right side (cf. Fig. 1) shown in Figure 2, it is seen that the surface of the collar toward the pulp is covered by a high epithelium with tapered cell bases between which other elongated epithelial cells are inserted. Cell division in the collar is limited to such cells, the nuclei of which are arranged in several layers. Immediately next to the barb-forming groove (ramogenous zone, *r.z.*, Fig. 2) these cells contribute to the zone in question. Next to the umbilicus they are continuous with the regeneration cells at the bottom of the follicle. But the great mass of cells of the collar beneath the high epithelium is concerned in axial growth by formation of the sheath of the feather germ. Typical sheath cells, recognizable cytologically, are formed beneath the dividing cells; they elongate, swing through an angle of about ninety degrees, undergoing keratinization, and form the sheath in the manner indicated in Figure 2.

The details of this interesting process will not be described here. It is sufficient to note in this place how relatively enormous a part of the activities of the collar are employed in the formation of the very dense, highly resistant, and strong sheath. It is from the sheath, in process of growth during the entire regeneration of the feather, that the energy and the mechanical support for continued axial growth are derived. The forming barbs are carried out with it in the manner described in Part C. The shaft has its own basis of growth in the collar, and its rate of growth is conformable to that of the sheath. The pulp is proliferated from the feather papilla (Fig. 1, *P.*) at a rate determined by the general axial growth.

B. RADIAL GROWTH

The increase of diameter of the collar during regeneration (radial growth) is of the order of 50 per cent in breast feathers during regeneration from the ninth to the fortieth day; and considerable reduction occurs in the last stages of growth. It is mentioned only for the sake of consistency because it does not appear to have any very definite relation to the problems under consideration.

C. ORIGIN AND GROWTH OF THE BARBS AND THEIR TANGENTIAL MOVEMENT

We stated in the first paper of this series (Lillie and Juhn, 1932) that two stages may be recognized in the origin of the barbs of the feather. In the first, which we estimated to include about twenty-five apical barbs on each side (in the breast feathers of the Brown Leghorn), the barbs appear to arise practically simultaneously. These barbs are placed vertically in the germ, as shown in Figure 3, Plate I, of our previous paper, thus showing absence of tangential movement as described below. Thereafter, we maintained, the origin of barbs is strictly serial from the ventral (antirhachis) region of the collar, so that each more apical barb of the definitive feather, situated more dorsally in the germ, is slightly older than the next basal. To this principle we still adhere as fundamental. Such variations as will appear beyond are consistent with the conception of ventral origination in the collar, which is a double gradient system as clearly proved by the hormone experiments described in our previous paper.

Our present conceptions demand a broader terminology for the ventral barb-originating region. The term "ventral triangle," which we originally used, will continue to be used in the restricted sense proper to it as descriptive of the arrangement of barbs immediately after their origin with bases divergent and apexes meeting in the mid-ventral line (Fig. 7, *et pas.*). This configuration, however, is due to the occurrence of tangential movements of the bases of the barbs (sec. C, 2). We shall therefore introduce the term "ventral locus" to designate the precise locus of origin of new barbs which is situated in the collar at the base of the ventral triangle when this configuration exists. When tangential movements are active, the ventral locus is highly restricted. Just preceding the formation of the after-feather, and for a short time after, it broadens out, tangential movements cease, and the conformation known as the ventral triangle disappears, to be replaced later by two (Figs. 12–17). Speaking theoretically, the ventral locus is the area between the ventral ends of the lateral collar gradients. The time of the original localization of the ventral locus is not strictly known, but it becomes clearly defined during the formation of the barbs of the tip of the feather. The term "ventral field" is used for the region in general, of which the ventral locus is the center.

We shall deal first with the movements that affect the disposition and growth of the barbs, and afterwards with their cellular origin.

1. AXIAL GROWTH OF THE GERM IN RELATION TO THE BARBS

The forming barbs are carried passively away from the collar by the axial elongation of the germ, effected as we have seen by the development of the sheath. The fact that they nevertheless maintain contact with the collar is explained by their continuous basal growth at the expense of the cells of the ramogenous zone immediately adjacent to the superior lip of the collar. We shall assume that the relation of the barbs or of any portion of the barbs that is once organized is fixed in relation both to the sheath externally and to the pulp internally; that is, there is no shifting of developing barbs on sheath or pulp. The consequence of these principles is that each formed locus of any barb is carried strictly axially during the entire period of its formation (cf. Fig. 3).

2. TANGENTIAL GROWTH IN RELATION TO THE BARBS

The term "tangential growth" ("tangential movement" of Juhn and Fraps) relates solely to the barbs; it includes the apparent movement of the barb bases from the ventral locus to their junction with the shaft (Fig. 3) and the growth forces responsible for this movement. In our former paper we interpreted this movement as due to a movement of the entire collar directed dorsally from the mid-ventral line and its utilization in formation of the shaft by total concrescence; growth centers for the barbs were presumed to be located in the collar and to be carried along by the moving halves of the collar, which thus dragged the forming bases of the barbs along with them. We can no longer support this interpretation *in toto;* in place of concrescence of the entire halves of the collar to form the shaft, we are concerned with concrescence of derivatives of the collar, which we now regard as itself a fixed system, to form its lateral surfaces only (cf. Fig. 9); and in place of postulating barb centers within the collar we regard the basal growth of the barbs as due to their own intrinsic utilization of material of the ramogenous zone derived from the collar.

The net result of the principles concerning the relations of axial and tangential growth to the formation of the barbs may be expressed in a diagram (Fig. 3).

At any given time, however (cf. Fig. 7 and other photographs of split preparations), a large series of successively formed barbs is present. In such a complement it follows from the foregoing principles and the serial order of origin of barbs from the ventral locus, (*a*) that the length of a barb in the germ as given by the axial position of its apex with reference to any complement of barbs simultaneously present accurately reflects its time of origin, (*b*) that in the absence of tangential movements the orientation of a barb in the germ will be strictly axial or vertical, and conversely, that the obliquity of a barb or of any of its axial parts in the germ is a measure of the rate of tangential motion with respect to axial growth, which may be treated as a constant.

3. BARBS OF IRREGULAR LENGTH

In accordance with the principle of strictly serial order of origin, all the barbs of any simultaneous complement should be graded in order of increasing length from the ventral locus to the shaft along the collar; but this is not always the case. Barbs of irregular length, longer than those adjacent to them on the shaft side, occasionally occur, and these are more frequent in the later stages of regeneration of the feather during the formation of its fluffy part, and especially during the time of origin of the after-feather.

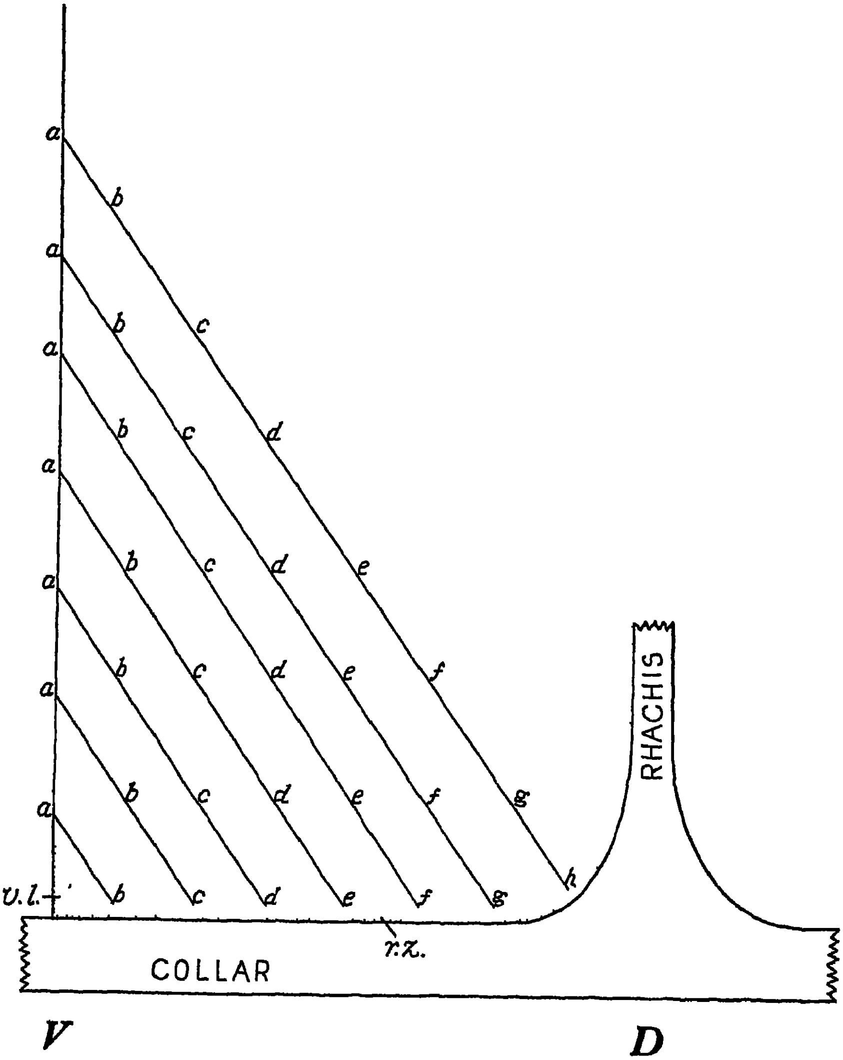


FIG. 3.—Seven successive positions of a single barb during its growth. Successively formed points at the base, *b*, *c*, *d*, *e*, *f*, *g*, and *h* are carried vertically at an equal rate, owing to uniform axial growth at each of these transverse levels. Owing to tangential movement, the successively formed basal points move toward the rhachis. The oblique orientation of the barb is thus due to a parallelogram of axial and tangential forces, which in the diagram are assumed, as one limiting case, to preserve a constant ratio during the entire formation of the barb. The apex of the barb, *a*, maintains its position at the mid-ventral line. *D* = dorsal; *V* = ventral; *r.z.* = ramogenous zone; *v.l.* = ventral locus. ×30.

The mode of origin of such barbs is as follows: Inasmuch as tangential movements are away from the ventral locus on both sides of the collar, there is a dead center at the ventral locus itself. Any barb that forms in this dead center is carried vertically by the axial growth of the germ (Fig. 4). In the meantime, other barbs are formed that participate in the tangential movement from the start. Subsequently, the barb in the dead center is caught up by the tangential movement, and therefore takes a place more ventral than barbs of later origin which have participated in the tangential movements from their start, although it is longer than they. Such barbs, while in the germ, always betray their origin by a relatively abrupt angle between the vertical part remaining in the dead center and the oblique part formed after participation in the tangential movements (Figs. 5, 6, *et al.*).

It is evident that the probability of formation of barbs of irregular length will depend upon the extent of the dead center corresponding to the ventral locus. During the formation of the vane of the feather the ventral locus is particularly restricted, but at the time of formation of the after-feather it becomes much broader, a condition that will be fully described beyond. Barbs of irregular length are very rare in the vane of the emerged feather, but are the rule rather than the exception in the apical part of the after-feather.

Hosker (1936, pp. 180–81) interprets the occasional occurrence of barbs of extra length on one side of the vane as indicating that "the ventral point is not fixed, but in this region of plasmatic growth, a barb which lay nearer to the left side of the feather germ might grow towards the right side of the rhachis." Such a denial of principle is fortunately unnecessary. It is impossible to visualize how a single barb can step out of the ranks at one place and rejoin them at another; but if a transference of the ventral locus from one position to another is meant, whole series of barbs, and never single barbs, must be involved.

4. CELLULAR BASIS OF ORIGIN AND GROWTH OF THE BARBS

The barbs originate and grow from a narrow zone of loosely arranged cells immediately adjacent and apical to the collar, which we shall call the "ramogenous zone." The rhachis interrupts this zone dorsally, but it is otherwise a continuous narrow ring (Fig. 3). It is located in the angle formed by the collar and the vertical walls of the feather germ (Fig. 2). The cells composing it appear to be derived by continuous proliferation of the adjacent (apical) margin of the collar; they are smaller than those of the collar epithelium, discrete and rounded; intercellular spaces produce the appearance of a network of strands; the epithelial surface next the pulp is here devoid of a basement membrane. The nuclei of the cells form about eight to ten layers, and karyokinesis may occur at any level. Transverse sections show well the reticular character of the ramogenous zone (Fig. 8). The barb septa disappear in this zone, which is entirely unsegmented, and all stages in the building-up of the bases of the barbs from the cells of the ramogenous zone can readily be seen. At some time we propose to publish a more detailed account of the foregoing interesting relations.

The ramogenous zone is the only part of the epithelial constituents of the feather germ which is not structurally coherent. It alone is structurally adapted to the execution of the tangential displacement of the bases of the barbs.

5. THE ORIGIN OF BARB APEXES

The origin of new barbs, i.e., of barb apexes, like their growth, is always from the ramogenous zone, but it is confined to the ventral locus, the extent of which is subject to some variation as we have seen (cf. p. 436). This locus is situated between the two gradients of the collar, and it is a tempting thought that its extent is a function of the steepness of the gradients. During the greater part of the growth of the feather it is surmounted by a triangular organization (Fig. 6) of cells of the ramogenous zone with the apex directed distally. The base rests on the collar, and the sides are split off as new barb apexes.[1] The latter thus have a considerable angle of divergence which is preserved as the combined forces of axial and tangential growth transport them in the manner already described (Fig. 3). In the region of the ventral locus the process of forming new barb apexes is then repeated. Right and left barb apexes need not be formed simultaneously. These processes have a practically invariable form, except during the formation of the apex of the vane and of the after-feather.

The barb apex once formed in the manner just described appears to act as a center of organization of the cells of the ramogenous zone; as it moves toward the shaft it continues to utilize cells of the ramogenous zone, thus availing itself of cells of all levels of the physiological gradient. The point at which growth ceases and its union with the shaft is complete is rather difficult to identify; cells of the ramogenous zone occurring between the bases of the growing barbs are carried with them on the lateral surfaces of the growing shaft. They are responsible for the origin of barbules arising directly from the shaft.

6 ANALYSIS OF TANGENTIAL MOVEMENT

Axial growth and movement is readily understood in principle on the basis of cell divisions and movements. It is not so with tangential movement, for there is no distribution of cell divisions and no evidence of streaming of cells in the direction of the movement to account for it. Various views are theoretically possible.

a) It might be supposed that an original oblique orientation of the part of the barb first formed persists and controls the direction of organization of all its later parts. This would approximate Hosker's interpretation, as I understand it. (Hosker, 1936, p. 176). This, however, would not hold for the apical barbs which are at first vertical (Lillie and Juhn, 1932), nor would it hold for the barbs forming the apex of the after-feather which are also vertical (see p. 445), and it would not account for definite changes in orientation during development, which are especially noticeable in the formation of barbs of exceptional length (p. 437). An essential part of the explanation of tangential movement must be found in conditions external to the forming barb as such.

b) A second theory might be based upon a reaction of the forming barb bases to the physiological gradients, which are evidenced in general by a high threshold of reaction to hormones next to the ventral locus corresponding to the barb apexes and a threshold diminishing in a dorsal direction along the collar to junction with the shaft. The theory would be in general terms that tangential displacement follows the gradient, and that its rate and hence the obliquity of the forming portion of any barb is proportional to the steepness of the gradient. This would be consistent with the vertical orientation of barbs in the "dead center," i.e., the place where the opposite collar gradients are in equilibrium. It is believed that a consistent theory might be formed upon this basis.

c) A third theory might be based on the postulate that successive formations of new barbs in the ventral locus act mechanically as a *vis a tergo* pushing the barb bases dorsally. This is the most obvious interpretation. However, the force seems inadequate to control the entire complement of barbs existing at any one time, unless it is supposed that such a purely mechanical factor is supplemented by a physiological relationship, such as is suggested in the second theory.

---

[1] Many feather germs, notably of the primary flight feathers, have strikingly asymmetrical ventral triangles, though symmetrical sides are more usual.

It is to be noted, with reference to apical barbs of both main and after-feathers, that the origin of tangential movements is synchronous with the origin of the shaft (cf. Figs. 12, 13, 14). It is also obvious that continuous axial growth of the shaft is a necessary condition for continuation of tangential movements of the forming barbs. These processes have a certain necessary association and correlation. The growth of the shaft has thus also to be taken into account in the analysis of tangential displacement.

D. THE FORMATION OF THE SHAFT AND THE ROLE OF CONCRESCENCE

In our previous paper (Lillie and Juhn, 1932) we ascribed the origin of the shaft simply to concrescence of the lateral halves of the collar. Renewed study has shown that the matter is not so simple; indeed, four longitudinal components may be distinguished throughout the extent of the shaft, a ventral and a dorsal forming the central axis, and two lateral components which fuse with the central axis.

The origin of the apex both of the shaft and also of the aftershaft is always indicated first by a properly located projection from the collar (Figs. 13 and 14, and Fig. 3 of our 1932 paper). Before this time the barbs are vertical with reference to the germ (cf. Figs. 12 and 13), and the bases are not in any way fused. As the shaft primordium elongates, it carries the apical barbs at first, and later, successive barbs with it. The bases of these barbs and the ramogenous material between them contribute to the lateral surfaces of the shaft primordium, as is very clearly demonstrated by the barbule-forming cells incorporated in this material.

The median part of the shaft which thus arises independently of the barbs has ventral and dorsal components of separate origin. The ventral component is derived from the collar epithelium which extends also in an active state along the ventral surface of the shaft for a certain distance until the smooth keratinized ventral surface is differentiated. In its actively dividing region it produces the cells that differentiate into the medullary cells of the shaft. But the keratin of the dorsal surface, which is separated from that of the ventral surface by the medulla and the lateral components, is derived from cells of independent origin placed between the medulla of the shaft and the feather sheath. Beyond saying that the cells in question are closely associated with the inner layer of the feather sheath, we do not wish to commit ourselves as to their origin.

The median part of the shaft arises from a spreading base in the collar (Fig. 7), which is also characterized by a much less massive development than the remainder of the collar (Fig. 8). The spread of the base indicates a central concentration of material in the formation of the shaft itself. The disposition and orientation of the melanophores which lie beneath the collar epithelium also indicate a lateral source of origin for those which enter the shaft (Figs. 7 and 17).

We conclude, then, that a process of concrescence is concerned in the formation of the lateral surfaces of the shaft, and that there is some concentration of material toward the center in the formation of the median part of the shaft. In any event, the median part of the shaft is of entirely different embryological origin from the barbs.

The diagram (Fig. 9) summarizes certain features of the foregoing description.

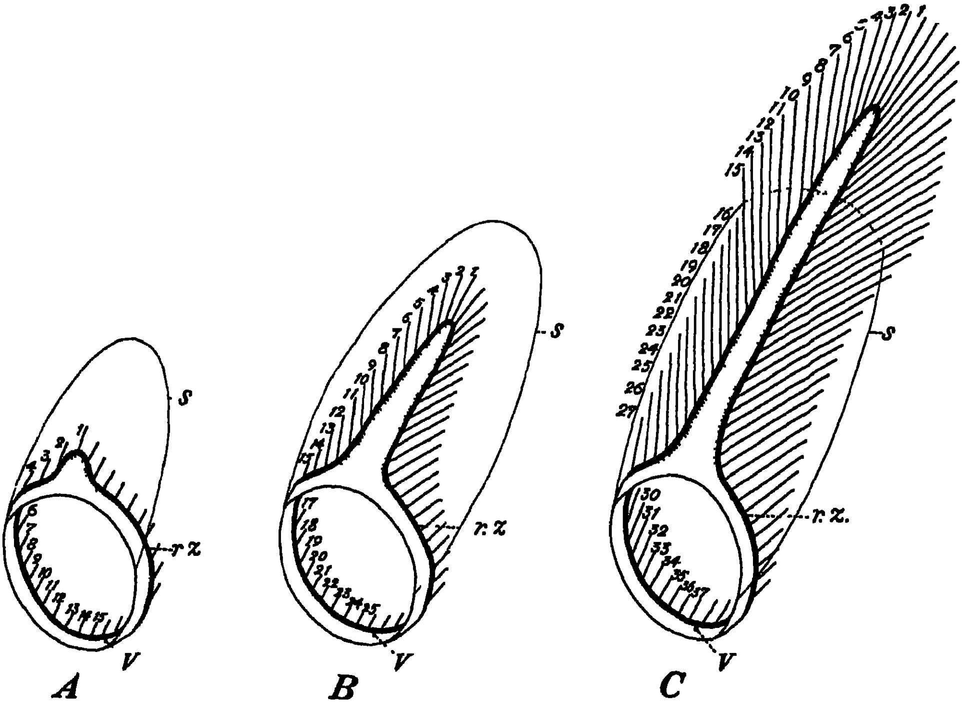


FIG. 9.—The diagrams represent three stages in the development of the feather. The two halves arise from a ring of embryonic cells, the collar, sunk in the feather follicle, and surrounding the base of the feather germ; the ridges or rami after the first complement (diagram *A*) arise in the ventral locus (*V*) grow at their bases from cells of the ramogenous zone (*r.z.*) adjacent to the apical margin of the collar, and simultaneously move tangentially on each side to the rhachis, carrying with them cells of the ramogenous zone to form the lateral surfaces of the rhachis As the axis of the rhachis (stippled) grows out from the dorsal region of the collar it carries the completed barbs with it. As a result of the tangential movements of the bases of the barbs, space is constantly provided at the ventral locus for origin of new barbs which arise serially on each side of the ventral locus (diagrams *B* and *C*, 16–37). The sheath (*s*) arising from the proximal lip of the collar (umbilicus) is ruptured apically after a certain stage (*C*).

At any given time the collar is beset with a series of forming barbs on each side which decrease in age from the rhachis to the ventral locus, and in state of development from the completely formed barbs, nos 1–25 (diagram *C*) to mere apical rudiments ventrally, nos. 35, 36, 37 (diagram *C*).

The order of formation of the shaft is apicobasal, and the order of age of the barbs the same. Similarly, in each barb the apex at the margin of the feather is the first formed and the central end attached to the shaft last. Thus there are two time gradients in each feather from apex to base along the shaft and from margin to center along the barbs. (Modified from Juhn, Fig. 43, ch. xiii, *Sex and Internal Secretions*, Baltimore, 1932.)

E. SUMMARY

1. The principle changes in our 1932 account which we have made are as follows:

a) Origin of the shaft: The principle of concrescence is limited to the formation of the lateral surfaces of the shaft.

b) Origin of the barbs: We have given up the principle of individual growth centers situated in the collar.

c) The explanation of the physiological gradient along the axes of the barbs evidenced by a rising threshold of reaction to thyroxin and female hormone from base to apex, or in the germ from dorsal to ventral surfaces, which was given in our 1932 paper, also requires modification. We are now uncertain whether the descending physiological gradient is correlated with a descending rate of growth of the barbs from apex to base. In any event, the gradient of growth-rate must be much less steep than was postulated.

2. The main additional principles that we have stated in the present paper are as follows:

a) The collar as such is a *fixed* double gradient system, the intensity of which is subject to quantitative variations during the development of the feather.

b) Axial growth of the germ as a whole is approximately uniform at transverse levels; however, it may vary at different axial levels, i.e., in time.

c) The sheath of the feather germ developing from the collar is the principal mechanical supporting element of axial growth.

d) The ventral locus situated at the "dead center" between the two gradients is subject to the stated variations in extent, relating especially to the origin of the after-feather (*q.v.*).

e) The apical segments of barbs act as organizers of ramogenous material arising from the collar; this conception replaces the theory of growth centers in the collar.

III. THE AFTER-FEATHER

A. INTRODUCTION

The general principles of this section were stated by us in a preliminary paper (Lillie and Juhn, 1937; see also Lillie, 1937).

The after-feather of fowl corresponds in length to a somewhat variable portion of the fluffy region of the contour feathers, never extending into the region of the vane. It emerges, as is well known, from the superior umbilicus, and its ventral surface is applied to the ventral surface of the main feather. It is well developed in connection with unspecialized contour feathers but is practically absent, or represented at most by a few barbs, in the flight and tail feathers. In the neck hackles it is absent or rudimentary anteriorly, but shows a gradually increasing development to a typical condition next to the cape and back. Its distribution in birds in general is given by Chandler (1916). Our account of its development will be confined to the breast and saddle feathers in fowl.

In these tracts it comprises the same parts and elements as the corresponding part of the main feather. Its parts measured from apex to base are synchronous in origin with the identical transverse levels of the main feather and partake of the same general fluffy structural characteristics. It is not as broad as the main feather, and its length relative to the main feather varies considerably, apparently in relation to its position in the feather tract or field. Reference to Figures 10, 11, and 18 will be sufficient for purposes of general orientation.

The study of the development is based upon over five hundred total preparations and a large number of serial sections, including all important stages. The early stages will be classified by the total number of barbs of the after-feather formed or in process of formation. After the formation of the hyporhachis, which occurs usually at or about the stage of six barbs, they are, of course, divided into right and left series approximately equal in number on the two sides.

The sex of the bird, the tract concerned, and position within the tract are intrinsic variables affecting the time of beginning of the after-feather reckoned from time of plucking. The following data are taken from accurately timed, systematic pluckings of breast and saddle feathers in a male and female Barred Rock respectively, involving over one hundred follicles: in the female the after-feather in the breast begins to form about the thirtieth day after plucking, when the length of the regenerating feather is from 4.6 to 5 cm.; in the male, formation begins a little later than in the female, at about thirty-five days. The saddle of the female is quite similar to the breast in these respects; but in the male the time of origin is much longer, about forty-three days. In the breast of both sexes the lateral feathers in positions 7, 8, and 9 (see Holmes, 1935) are later in beginning than those in positions 1–6; number 9 indeed may have only one afterbarb at a time when the average in numbers 1–6 is 42.

These observations suggest problems of considerable interest, especially in relation to field functions. However, they are mentioned here merely as a guide to the description of stages that follows. The foregoing data for the Barred Rock are controlled to a certain extent by other less complete series. Season of the year, condition of the bird, and repetition of pluckings affect the onset of regeneration, and hence the age reckoned from plucking.

The general plan of development of the after-feather may be readily understood by examination of total preparations. These are made by splitting the cylinder of epithelial cells surrounding the pulp on one side from the umbilicus longitudinally, spreading it out flat, pulp side out, and fixing it under pressure between two glass slides, the preparation is then dehydrated, cleared, and mounted without staining. Such a preparation affords a view of the interior of the feather germ and displays an astonishing amount of detail. The preferred lines of incision are near the dorsal surface, midway laterally, or near the ventral surface. The last is not suitable for the study of the after-feather. The constriction of the umbilicus (see Fig. 1) necessarily submits the region of the collar to considerable strain in this method of preparation, and it must be realized that the delicate bases of the barbs may thus be subject to some distortion. Allowance must be made for this in using such preparations, but it does not affect the subject under consideration in any essential way.

Figure 7 exhibits the basal portion of an entire lateral split preparation made about one week before the time of origin of the afterbarbs. The photograph is useful for general orientation, and attention should be directed especially to the conformation of the ventral triangle.

B. DEVELOPMENT

1. IRREGULARITIES IN THE VENTRAL LOCUS

Stages immediately preceding the actual origin of the after-feather are characterized by a greater frequency of barbs of irregular length. It will be remembered that these arise from the apex of the ventral triangle, remain in the dead center, and thus grow vertically for a variable period of time before being taken into the tangential stream. They are accordingly characterized by greater length than neighboring more dorsal barbs, and also by a rather abrupt angle between the vertical apical part and the oblique part formed after entering the tangential stream (cf. Figs. 4, 5, 6, and 12).

At this time also, and during early stages of the after-feather, other irregularities in barb formation appear quite commonly in the ventral region; barb apexes may be completely detached (see Fig. 6), bases of barbs may appear constricted, or two apexes may have a common base. Such abnormalities are frequent enough to indicate a considerable disturbance of the ventral region. This instability persists for a certain period during the early development of the after-feather, but disappears later.

2. THE ORIGIN OF THE FIRST AFTERBARBS

The formation of the after-feather is initiated by the origin of a series of vertical barbs from a broadened ventral locus (Fig. 12). In accordance with our interpretation of vertical barbs (p. 439), tangential movements have now ceased in this region, while axial growth continues. Also according to our interpretation of the relation of axial growth to length of barbs, the length of a barb is a measure of its age with reference to its neighbors. Accordingly it will be observed that the first vertical barbs do not arise in a regular sequence like their predecessors and successors. For instance, in Figure 12 the three afterbarbs are of increasing age from left to right. This irregularity in origin of the first afterbarbs is reflected in the apex of the after-feather.

It is interesting to recall that the apical barbs of the main feather arise also in a vertical position before any sign of the shaft appears, after which tangential movements begin and the bases become oblique as they join the shaft (Lillie and Juhn, 1932).

3. ORIGIN OF BILATERAL SYMMETRY OF THE AFTER-FEATHER

The origin of bilateral symmetry of the after-feather is associated with the appearance of the hyporhachis and the distinct delimitation of two ventral loci.

a) The two ventral loci are derived from the lateral margins of the broad ventral locus of the preceding stage. This may be interpreted as a process of division or budding of the original single ventral locus; its activity becomes concentrated at its margins which have been separated by the vertical afterbarbs. Each new locus has now the same bilateral activity as the original one, forming barbs right and left of its center (cf. Figs. 13–17). In terms of the gradient theory, there are now four gradients in place of two, directed specifically from each center toward the rhachis of the main feather and toward the hyporhachis. Each locus contributes to the right side of the main feather and the left side of the after-feather, or vice versa. The consequences of this will be considered later.

b) Bilateral symmetry of the after-feather itself is established by the origin of the hyporhachis in the approximate center of the vertical afterbarbs, when about six are present. In the entire preparation (Fig. 13) it appears as an unpigmented moundlike projection of the collar into the bases of the barbs, whose pigmented bases are entirely distinct from it. As it rises it carries the afterbarbs with it. It is plain, and must be emphasized, that the hyporhachis is an independent formation derived from the collar, and that the attachment of the barbs is secondary, as is also their contribution and that of the ramogenous material to its lateral surfaces.

As soon as the hyporhachis is established, tangential movements of the bases of the barbs toward it begin, clearly indicated by their oblique disposition. This is already to be seen in one of the barbs of Figure 13. In the stage of eighteen afterbarbs shown in Figure 14, tangential movement is already actively under way.

4. LATER DEVELOPMENT OF THE AFTER-FEATHER

Later stages in the development of the after-feather are shown in Figures 15, 16, and 17. In the last, which shows only its basal portion, the after-feather is near completion.

Study of these figures and of their captions will serve better than any detailed description in the text for a survey of the progress of events. The relations of the two ventral loci and their triangles to the main feather and after-feather, the elongation and attenuation of the hyporhachis, and the steady addition of new barbs to both feathers are especially to be noted. Throughout these stages tangential movement of barbs, reflected in the obliquity of their bases, is active in both directions from each ventral locus. After the period of irregularity at the time of origin of the first barbs of the after-feather, the ventral loci have settled down to machine-like regularity.

5. DISCUSSION

a) *The conception of collar gradients.*—The conception of gradients in the collar is based upon our experimental determination (1932) that the threshold of reaction to the female hormone or thyroxin in breast and saddle feathers rises in the germ from the dorsal (rhachial) extremity of the collar to the ventral locus, or in the case of the barbs from base to apex. There are thus two gradients corresponding to the two halves of the vane of the feather descending from the sides of the ventral locus in the germ to the sides of the rhachis.

The ventral locus determines the rate of origin of barbs; it thus asserts a kind of dominance over the entire process of feather formation. We shall present reasons below for believing that its dominance expresses itself also in special ways.

b) *Twinning in the origin of the after-feather.*—The two ventral loci associated with the development of the after-feather are derived from the material of one, the original ventral locus. The new ventral loci inherit, so to speak, an established gradient in the collar on the rhachis side of each and are related in similar manner to new gradients on the hyporhachis side of each. The twinning is thus obviously not a division of the whole germ system. But the results, described below, speak for a dominance of the whole system by the new loci.

The twin triangles are much closer to the hyporhachis than to the rhachis, especially in early stages of the after-feather (Fig. 15); and even in late stages (Fig. 17) they do not become equidistant. In the first case, the difference is of the order of eight to one, and in the latter more than two to one. A barb destined for the apical portion of the after-feather thus undergoes only one-eighth as much tangential movement during its growth as one destined for the main feather, but it attains from one-third to one-half its length (Fig. 11). On the principle of equal axial growth at all transverse levels of the germ, i.e., equal increments of length in equal periods of time, it must follow that tangential movement is slower on the hyporhachis side of the ventral loci. An interesting correlation of this will appear later in comparison of the barbs of the main feather and the after-feather.

In accordance with our previous interpretation, each of the new ventral loci must be regarded as the high point of a double gradient system which terminates in hyporhachis and rhachis, respectively. They thus enjoy a certain degree of independence, being blocked off from one another by the rhachis on one side and the hyporhachis on the other.

In the Barred Rock fowl (Montalenti, 1934) the initiation of the black bars begins at the ventral triangle and progresses toward the rhachis. When it is reached the white bar begins to form. Black and white phases of activity are not necessarily quite synchronous on the two sides of the vane. It thus frequently happens that in preparations one side next to the ventral triangle may be in the black phase and the other side in the white phase (cf. Fig. 5). The two sides are "out of step," so to speak. Similarly, the two ventral triangles of the after-feather stage may be out of step. In such a case the one in the black phase will be associated with asymmetrical black spots placed on morphologically opposite sides of the main feather and after-feather, but superimposed, owing to the face-to-face relationship (see p. 443).

This phenomenon makes it necessary to postulate dominance by the ventral locus in the process of pigment formation (development of melanophores), for the only thing that opposite halves of the vanes of the twin feathers have in common is precisely the common ventral locus. This conclusion is of interest not only in a physiological but also in a genetic sense, because Montalenti has shown that the rhythmicity in barring is a function of the genes, which are therefore shown in their turn to be controlled as to time of action by a physiological system, described by us in terms of a gradient hypothesis.

C. PATTERNS IN THE DEFINITIVE AFTER-FEATHER AND THE MAIN FEATHER

In most breeds of fowl, pigment patterns are not found in the after-feather nor in the corresponding part of the main feather. The Barred Rock is an exception, however, for the barred pattern extends in the best examples to the base (Fig. 10). This enables comparison of pattern in the twin feathers. The bars correspond in the two so closely that they very nearly superimpose in the natural position of the feather; the black and white phases in development have been synchronous in the twin feathers, or nearly so. Moreover, defects in barring are repeated in the twin feathers, frequently at least. As an example, the second black bar of the after-feather in Figure 10 is defective on the observer's left in both; this is, however, actually the right side of one and the left side of the other. In the few cases in which barring is absent, it is so in both partners, and the ground tone, white to dark grey, is the same in both.

But the most remarkable examples of twin asymmetry and its reversal of side in our material are found in the hybrids between Brown Leghorn and Barred Rock. Here they are also very numerous. In these hybrids typical barring never extends into the fluffy region of the main feather or, *a fortiori*, into the region of the after-feather, though parti-colored patterns may occur. The superimposed regions of the partners are always the same (Figs. 11 and 18), thus all white or all gray or black, or if parti-colored, the resulting patterns are the same in both. There are various types of parti-colored patterns. For instance, a black tip of an after-feather otherwise white corresponds to the level of the main feather where black passes into white (Fig. 18C). If a black spot is confined to one side of the after-feather it corresponds accurately in position and form to a black spot of the main feather, though on its morphologically opposite side. The figures and their descriptions will give a better account than much writing in the text.

But, like all other twin characters, there is never complete identity of pattern. The special interest of these twin feathers is that their development explains so many features not only of resemblance but also of difference.

a) Axial level resemblances are explained by the principle of equal axial growth of the germ.

b) The barbs of the main feather are longer than those of the after-feather, for they take more time to complete their tangential movements and have longer time in which to grow. It is of course the basal part of the barb of the main feather that is lacking in the after-feather.

The barb of the main feather in the region of the after-feather consists of two quite sharply distinct parts: a short basal region in which the barb proper is flattened in an apicobasal direction, thus presenting the flat surfaces toward its neighbors, and a much longer cylindrical part. The transition between the two is quite abrupt. The basal part possesses short stout barbules, and the distal part extremely long (about 6 mm.) hair-like barbules (cilia). In the after-feather the barbs resemble only the long, distal part of the barb of the main feather, and the barbules are also similar. The homologue of the basal part of the barb of the main feather is completely lacking in the case of the after-feather. The obvious explanation is that the growth of the afterbarb ceases too early for the development of this homologue. An interesting corollary of this is that the barbules situated on the sides of the shaft between barb attachments are short and stout in the case of the main feather, and long and filiform (cilia) on the hyporhachis. As a result of this situation, the pigment spots described are narrower on the after-feather than on the main feather and correspond to the marginal zone of the main feather.

c) The reversal of "sidedness" is a necessary result of the fact that when either ventral locus is in a different phase from the other, as in all the asymmetries, its effect is on adjacent sides of the twins which are morphologically opposite.

d) Two or three of the apical barbs of the after-feather have, strictly speaking, no homologues in the main feather. Homologous barbs cannot, in fact, arise until the two new ventral loci are definitively established. These barbs have thus a somewhat special character explained by their developmental history.

There are thus reasons for lack of complete identity of twin characters which are intrinsic and need not be referred to any environmental difference. The significant aspect is that the causes for the dissimilarities mentioned are developmental, not genetic, and that they can be identified.

LITERATURE CITED

CHANDLER, A. C. 1916. A study of the structure of feathers with reference to their taxonomic significance. U. of Cal. Pub. in Zoöl., 13:243–446.

FRAPS, R.M., and JUHN, M. 1936*a*. Developmental analysis in plumage. III. Field functions in the breast tracts. Physiol. Zoöl., 9:378–406.

———. 1936*b*. Developmental analysis in plumage. II. Plumage configurations and the mechanism of feather development. *Ibid*., 9:319–75.

HOLMES, A. 1935. The pattern and symmetry of adult plumage units in relation to the order and locus of origin of the embryonic feather papillae. Amer. Jour. Anat., 56:513–37.

HOSKER, A. 1936. Studies on the epidermal structures of birds. Phil. Trans. Roy. Soc. London, B, 226:143–88.

JUHN, M., and FRAPS, R. M. 1934. Pattern analysis in plumage. IV. Order of asymmetry in the breast tracts. Proc. Soc. Exper. Biol. and Med., 31:1187–90.

———. 1936. Developmental analysis in plumage. I. The individual feather: Methods. Physiol. Zoöl., 9:293–317.

JUHN, M., and GUSTAVSON, R. G. 1930. The production of female genital subsidiary characters and plumage sex characters by injection of human placental hormone in fowls. Jour. Exper. Zoöl., 56:31–61.

JUHN, M.; FAULKNER, G. H.; and GUSTAVSON, R. G. 1931. The correlation of rates of growth and hormone threshold in the feathers of fowls. Jour. Exper. Zoöl., 58:69–111.

LILLIE, F. R. 1937. The development of the after-feather: A process of twinning. Collecting Net, 12:125–29.

LILLIE, F. R., and JUHN, M. 1932. The physiology of development of feathers. I. Growth-rate and pattern in the individual feather. Physiol. Zool., 5:124–84.

———. 1937. The origin of the after-feather: A process of twinning. Science, 86:38–39.

MONTALENTI, G. 1934. A physiological analysis of the barred pattern in Plymouth Rock feathers. Jour. Exper. Zoöl., 69:269–345.

PLATE I

FIG. 1.—Feather germ of Brown Leghorn capon from the posterior part of the breast; 18 days' regeneration. *Frontal* longitudinal section in follicle. *A* = central artery of pulp; *C* = collar; *d* = derma; *Ep* = epidermis of skin; *F* = cavity of follicle; *P* = pulp; *Pa* = papilla; *r.s.* = ramogenous zone; *S* = sheath of feather germ. ×ca 22.

FIG. 2.—Collar and adjacent region from right side of Fig. 1. *r.c* = regeneration cells; note the connection of these follicular epidermal cells with the collar; they represent a special proliferation of the follicular epidermis from which in conjunction with the papilla the next regeneration will take place. When a feather is forcibly plucked it carries with it all the collar and some of the regeneration cells; the pulp is removed *in toto*, but the papilla remains in place. *B'bl.* = barbule cells; *ep.c.* = proliferating epithelium of collar; *r.s.* = ramogenous zone. ×*ca.* 184.

PLATE II

FIG. 4.—Ventral field of a split preparation (see p. 444) of a Barred Rock female from the left anterior breast (feather *l.b.a.*5) to show a central vertical barb in an early stage (cf. p. 439); 24 days' regeneration. *V* = ventral. ×50.

FIG. 5.—Ventral field of a split preparation of a Barred Rock female from the left anterior breast (feather *l.b.a.*9); the left side is in the black phase, and the right side in the white phase of barring. The third barb on the left consists of a vertical apical portion and an oblique basal portion with an abrupt angle between. To illustrate origin of barbs of irregular length see p. 437; 24 days' regeneration. ×50.

PLATE III

FIG. 6.—Ventral field, showing triangle and precise location of ventral locus, from Barred Rock male saddle (feather *l.s.c.*3). Stage just before beginning of after-feather. Barbs of irregular length, right and left; also other irregularities (cf. p. 444). ×*ca.* 100.

FIG. 8.—Transverse section of collar at stage of five afterbarbs (cf. Fig. 12). At this level the main mass of the collar is composed largely of early sheath cells. The feather germ from which this preparation was made was plucked, and the pulp was torn away from the collar on the right side. The section passes through the broad base of the rhachis (*Rh*) (cf. Fig. 7), which interrupts the thickened welt of the collar near its apical margin. A portion of the ramogenous zone appears on each side of the rhachis region (*r.s.*); in more apical sections it can be followed all the way around except in the region of the shaft (cf. p. 439). *A* = central artery; *V* = ventral.

PLATE IV

FIG. 7.—Entire width of base of total preparation split laterally to show relations of ventral field and rhachis. Hybrid capon (Barred Rock×Brown Leghorn); about one week before origin of after-feather; breast (feather *r.b.a.*9); *V* = ventral; *D* = Dorsal. ×30.

PLATE V

FIG. 10.—Barred Rock male: *A*. From left back, row *c;* ventral view with after-feather displaced; *B*. From left breast with after-feather similarly displaced.

FIG. 11.—Hybrid capon (Barred Rock×Brown Leghorn). Ventral view of base of feather with after-feather turned back. *A*. From right saddle, row *d;* main and after-feather all white; *B*. From right saddle, row *c;* main and after-feather with similar black spots; on the right side in the main feather and on the left side in the after-feather, the ventral surface being up in both.

PLATE VI

FIG. 12.—Ventral field in total preparation, stage of three afterbarbs (cf. p. 445); no hyporhachis. Barred Rock female, saddle (feather *l.s.d.*4); 40 days' regeneration. *A'f* = after-feather; first three barbs. ×85.

PLATE VII

FIG 13 —Ventral field in total preparation Usual symmetrical arrangement of six afterbarbs (*A'f*) with beginning stage of hyporhachis in the center, appearing as a low rounded projection from the collar Barred Rock female saddle (feather *r s d* 2), 40 days' regeneration ×85

PLATE VIII

FIG 14 —Ventral field in total preparation After feather (*A'f*) with nine afterbarbs on each side, and moundlike hyporhachis The two ventral triangles (*V'*), right and left of the after feather, are well differentiated Note the apparent fusion of the two central afterbarbs at the apex of the hyporhachis, and also the irregular lengths of afterbarbs on the right Barred Rock female saddle (feather *r s d* 4), 40 days' regeneration ×*ca* 73

PLATE IX

FIG 15 —Entire width of base of total preparation, lateral split, to show relation of after feather (*A'f*) and hyporhachis to main feather and rhachis About ten afterbarbs on each side Note arrangement of chromatophores at spreading base of rhachis Hybrid capon (Barred Rock×Brown Leghorn) breast (feather *r b d* 6), about 40 days' regeneration *V'*, *V'* = the two ventral triangles ×*ca* 24

PLATE X

FIG 16 —Ventral field in total preparation After feather (*A'f*) with about eighteen afterbarbs on each side Note especially further elongation and narrowing of hyporhachis Barred Rock female saddle (feather *l s f* 1), 37 days' regeneration *V'*, *V'* = the two ventral triangles ×*ca* 60

PLATE XI

FIG 17 —Entire width of base of total preparation split close to rhachis on left to show relations of after feather (*A'f*) and main feather More advanced stage of after feather than preceding figures Note relations of chromatophores in the collar to those in the rhachis Barred Rock female saddle (feather *l s e* 4), 37 days' regeneration *V'*, *V'* = the two ventral triangles ×30

PLATE XII

FIG 18 —Saddle feathers of hybrid capons (Barred Rock×Brown Leghorn), ventral views with after-feather turned back Identical patterns of after feather and main feather with reversal of side *A* Capon 229 saddle (feather *r s c* 3), vane pure Barred Rock type *B* Capon 198 saddle (feather *l s a* 1), vane pure Leghorn type *C* Capon 198 saddle (feather *r s a* 3), apical half of vane Barred Rock type, basal half Leghorn type

PLATE I

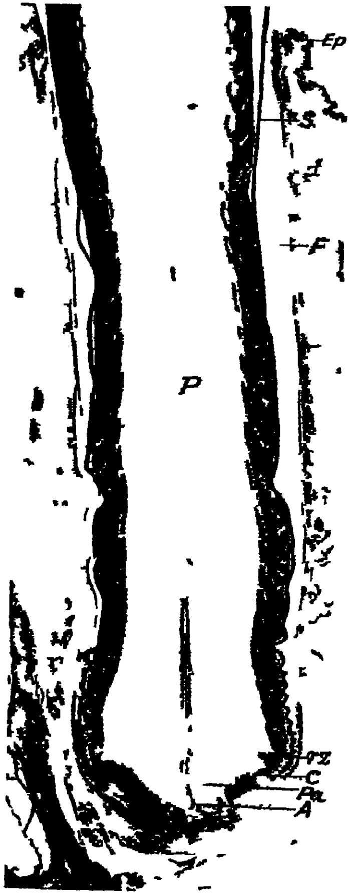
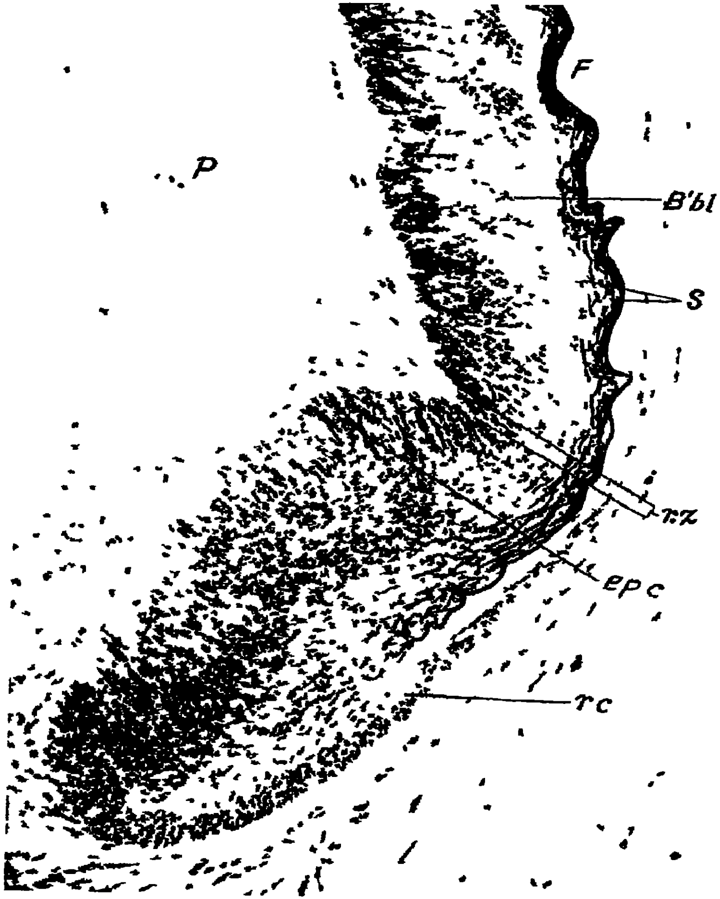


FIG I



[illegible]

PLATE II

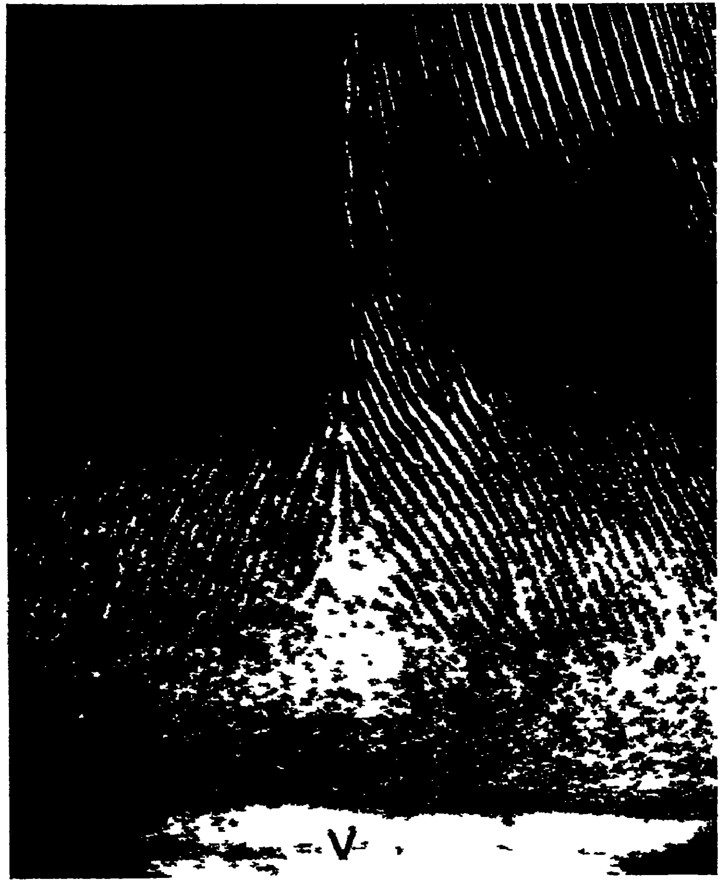


Fig 4

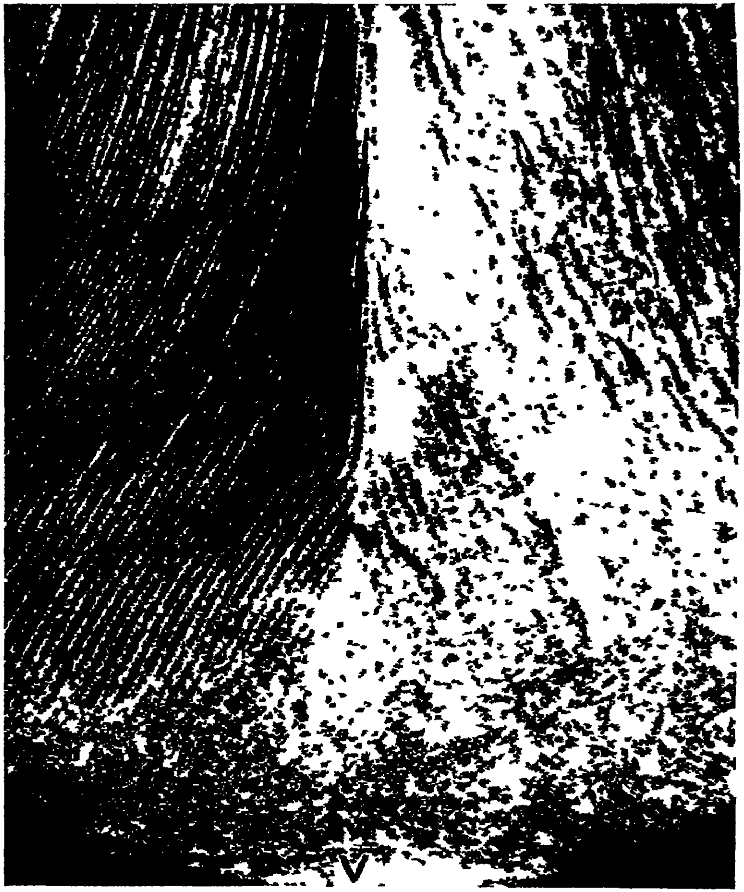


Fig 5

PLATE III

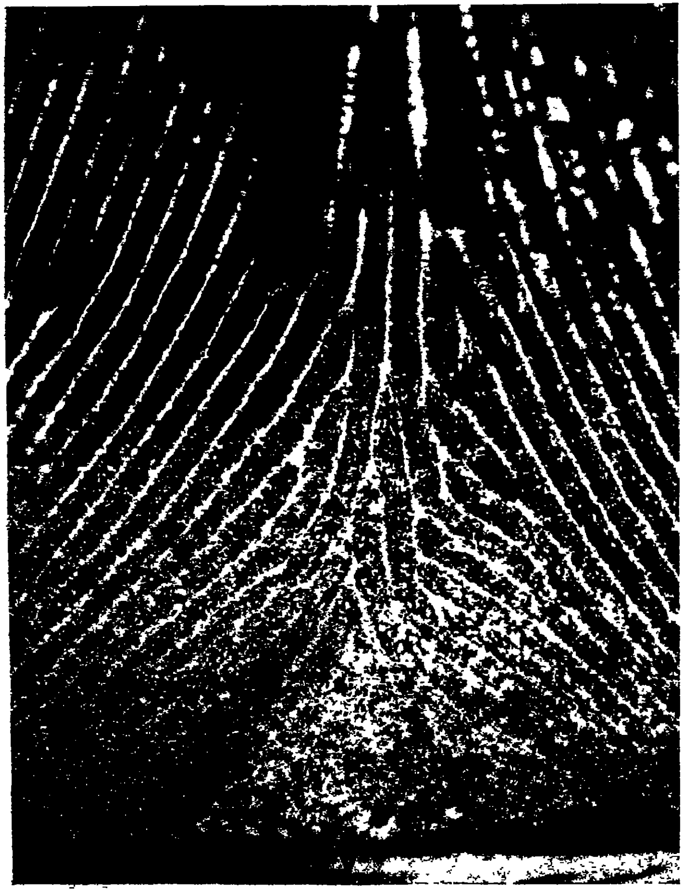


Fig. 6

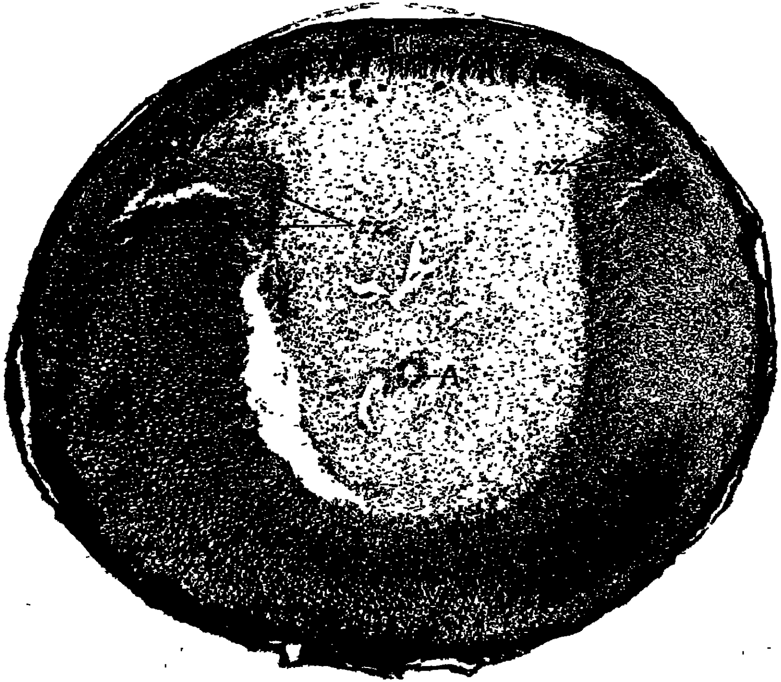
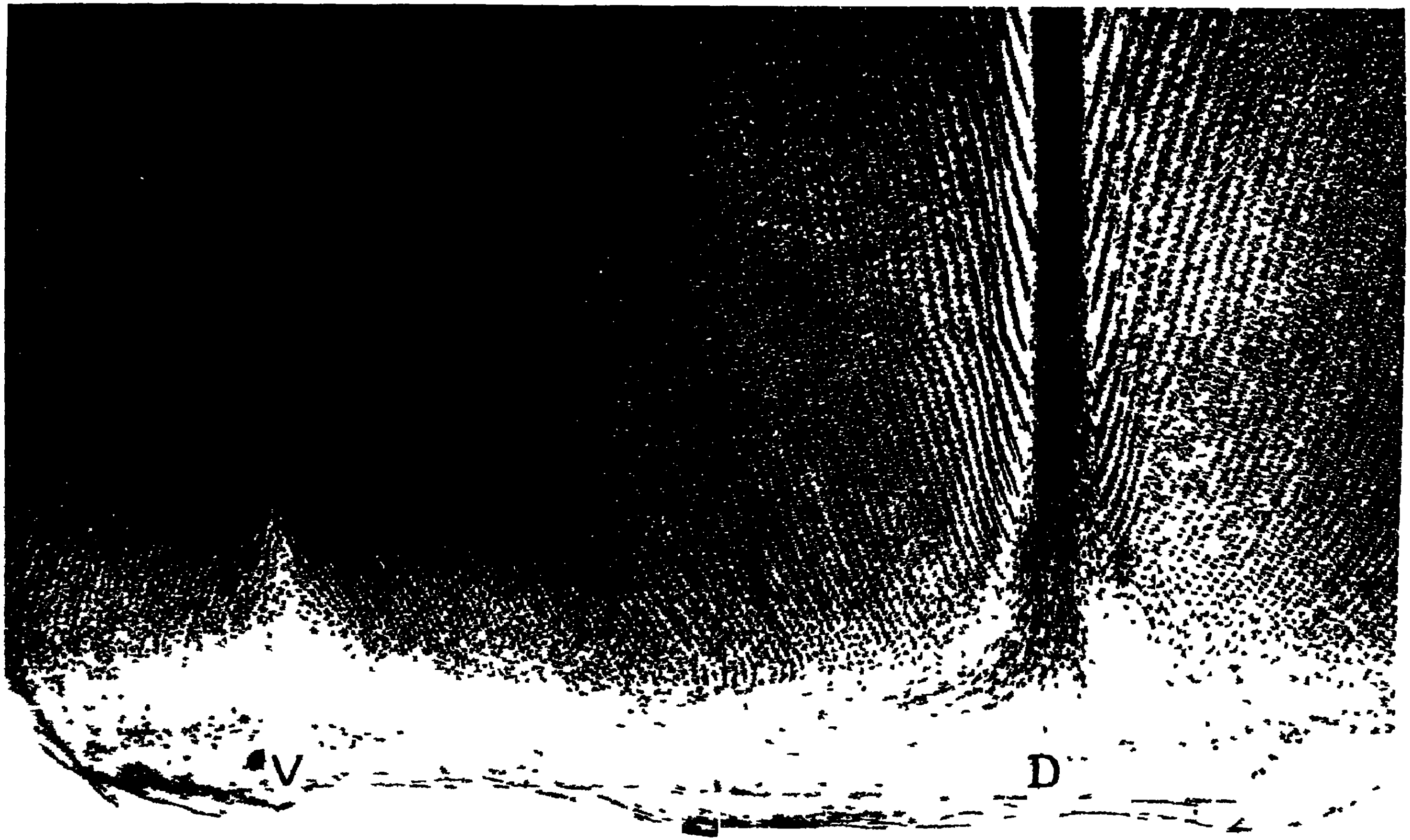


PLATE IV



Fig

PLATE V

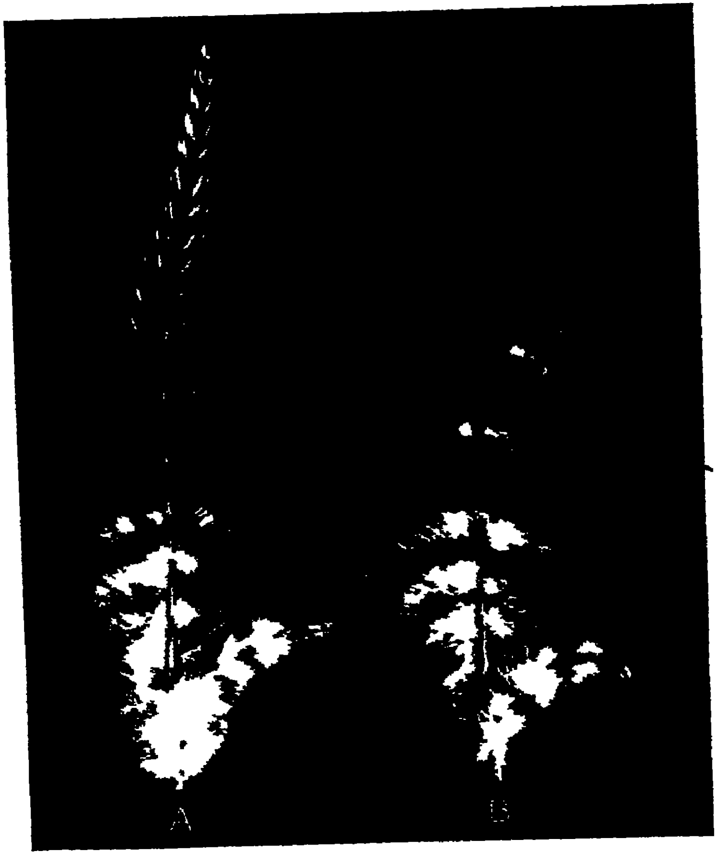


Fig 10

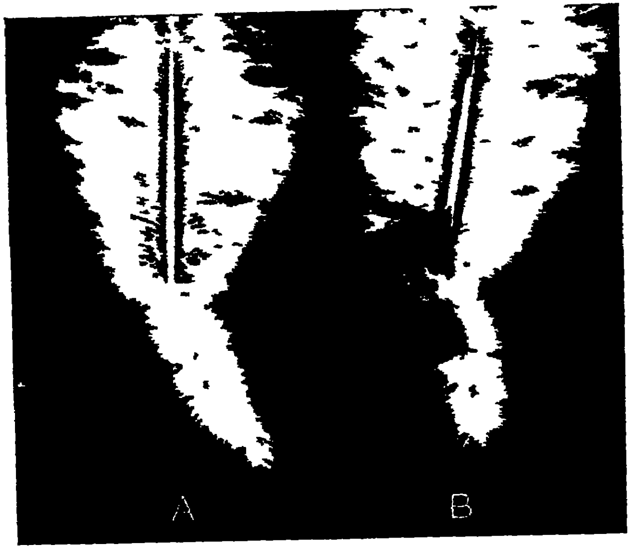


Fig 11

PLATE VI

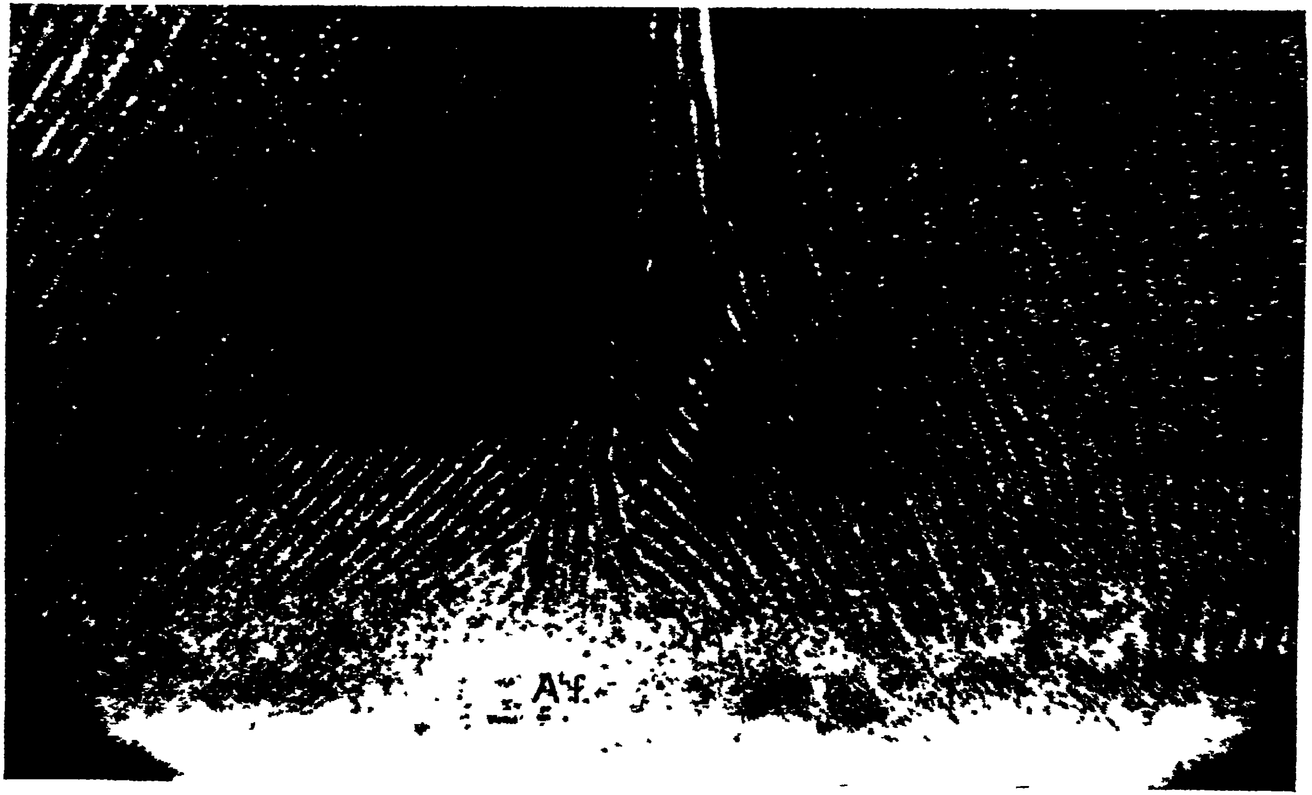


FIG. 12

PLATE VII

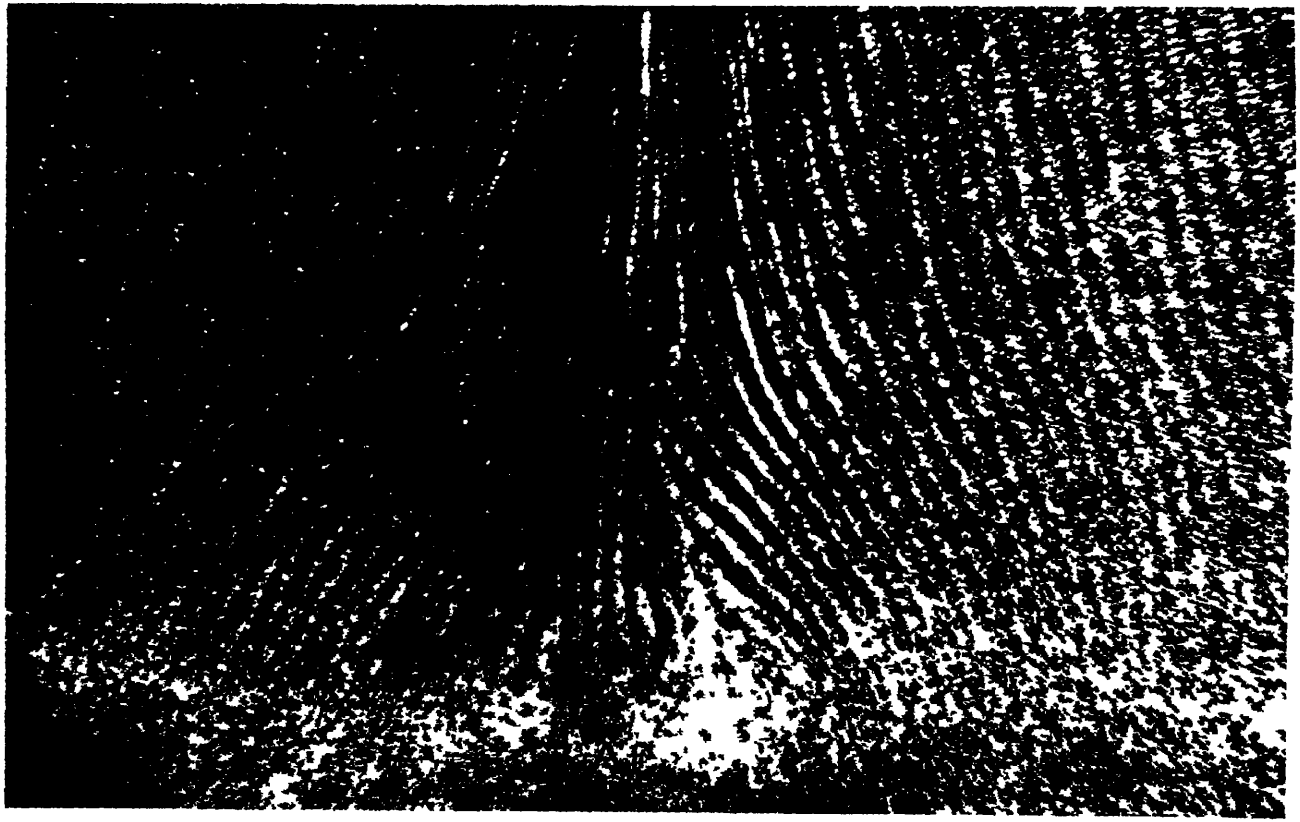


FIG. 13

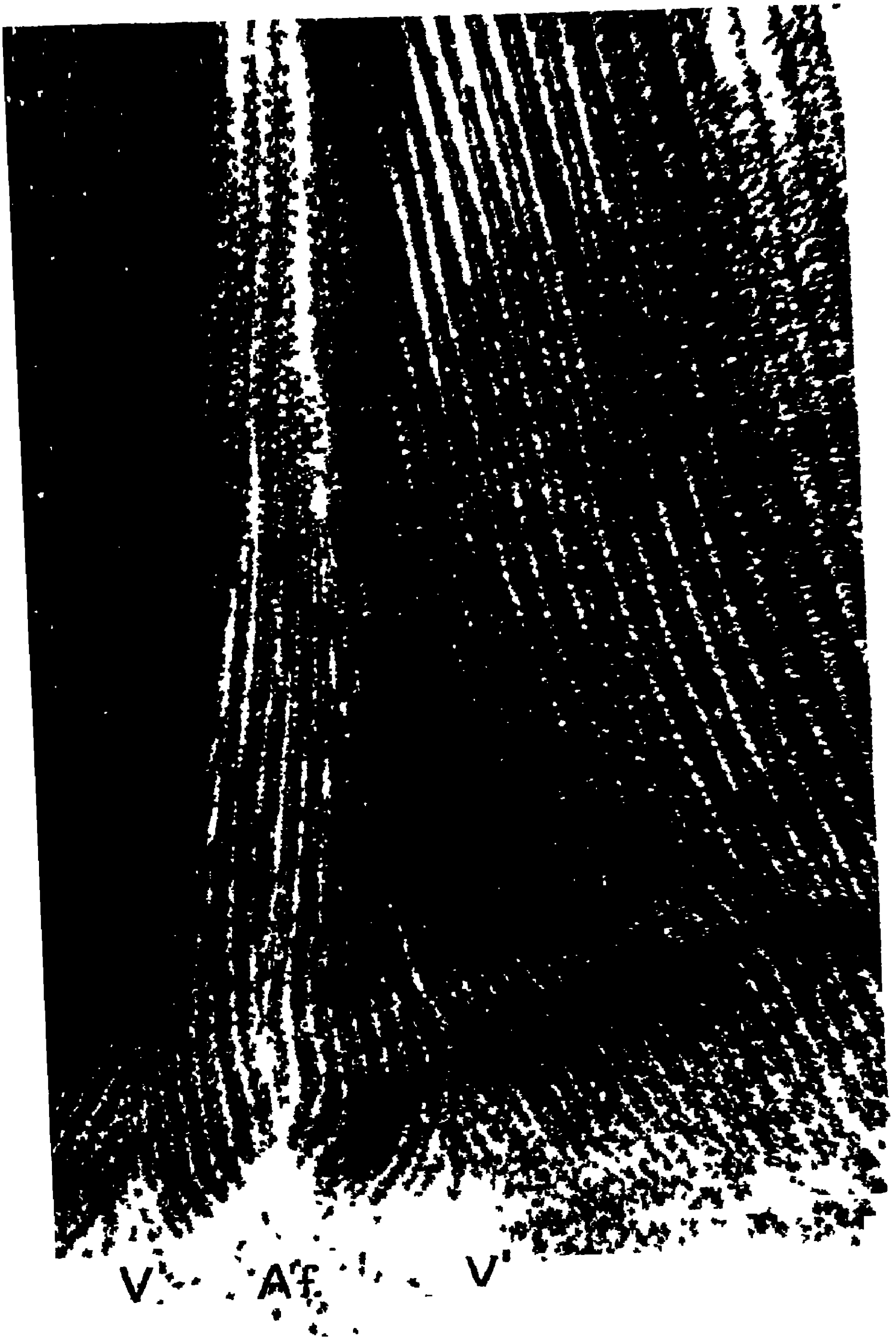


Fig. 14

PLATE IX

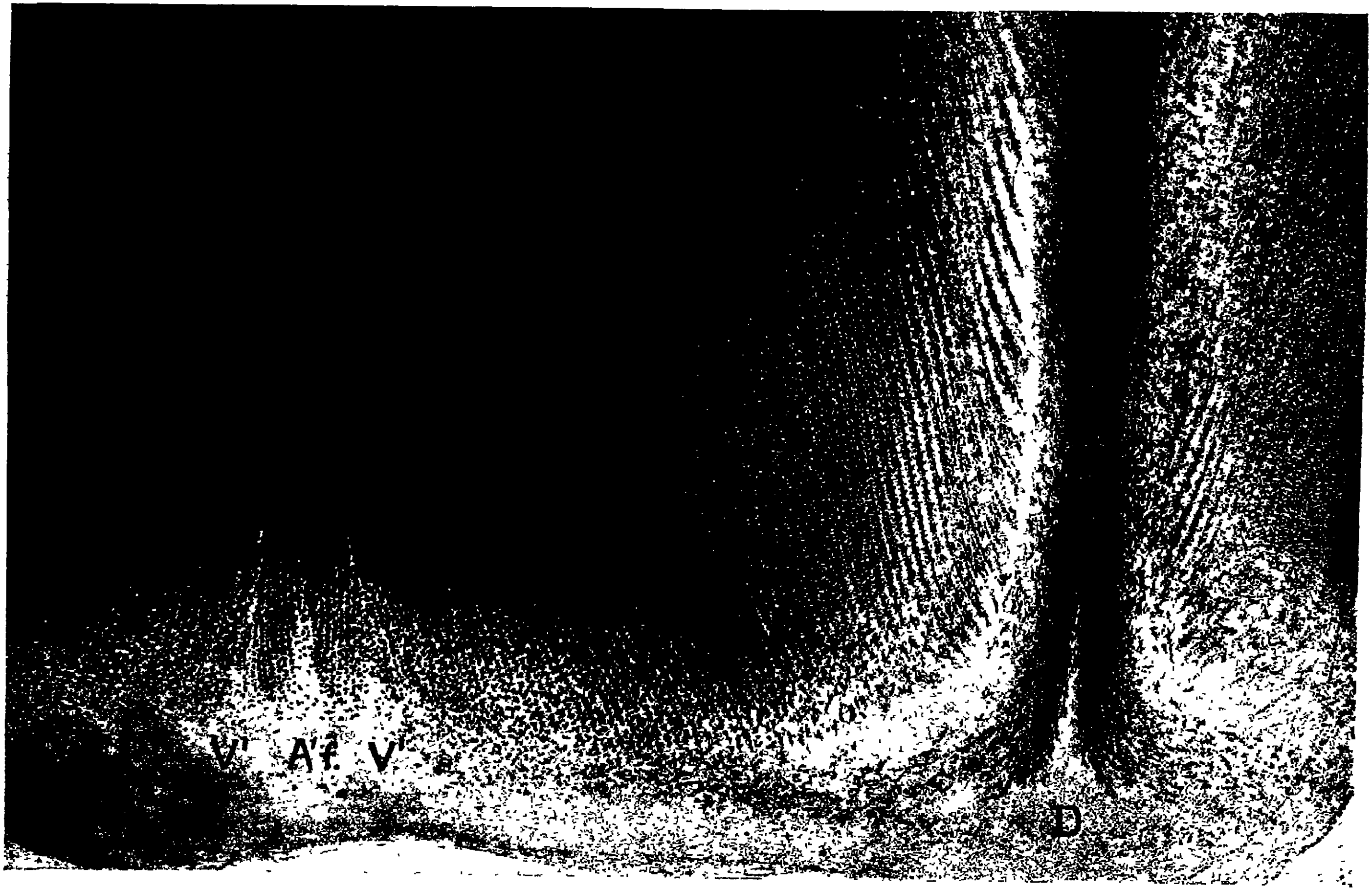


FIG. 15

PLATE X

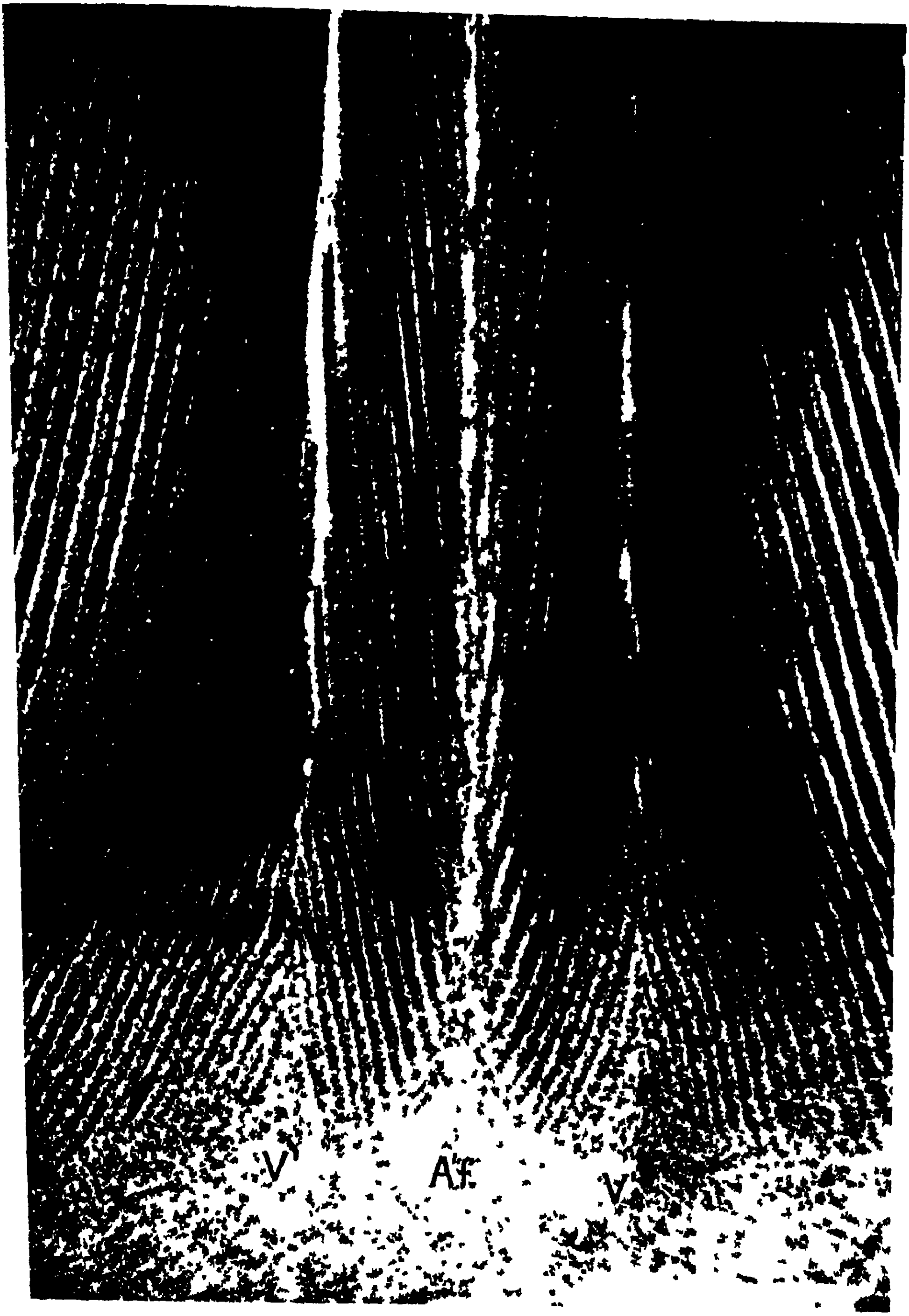


FIG 16

PLATE XI

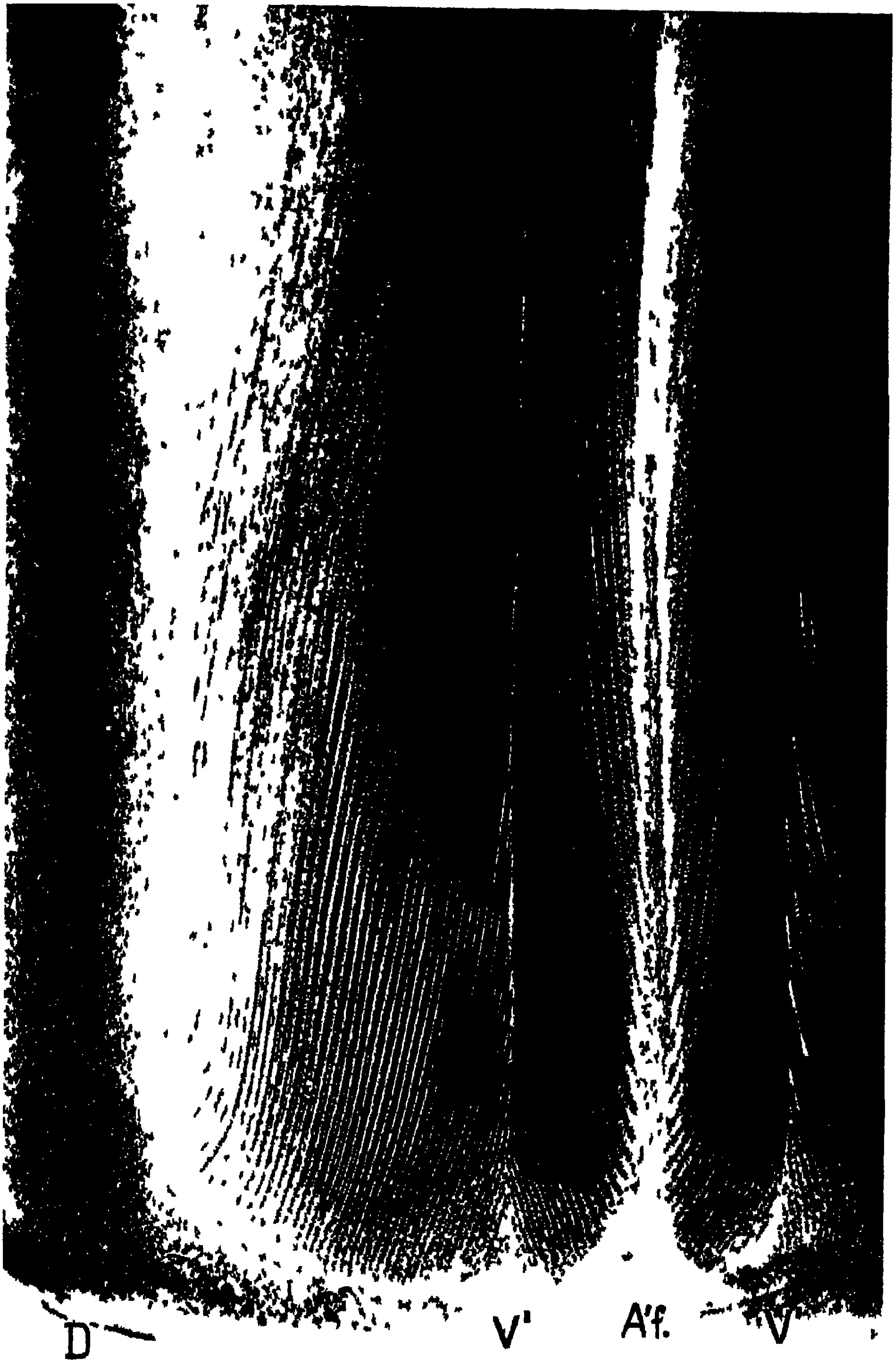


Fig 17

PLATE XII

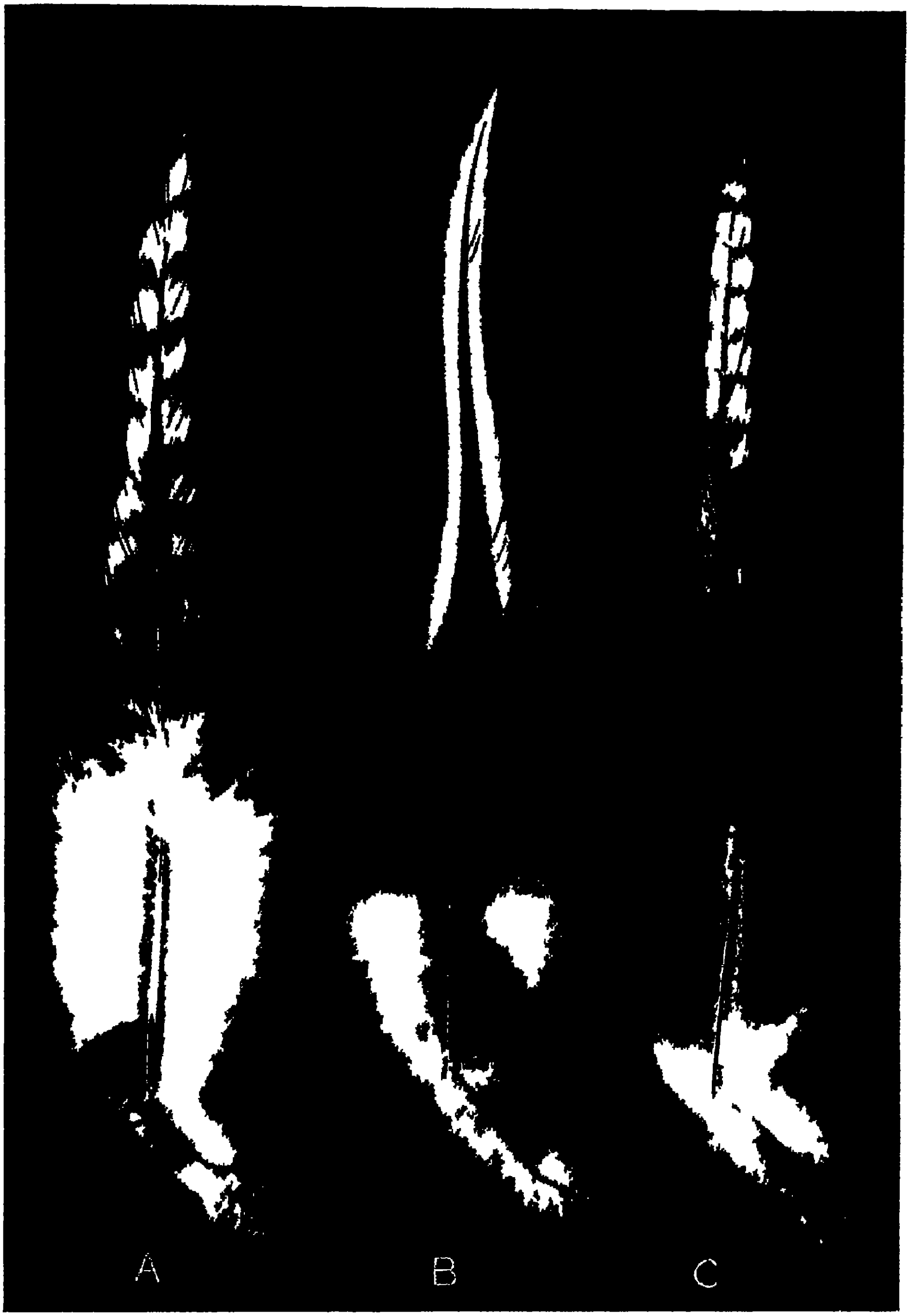


Fig 18

INDEX TO VOLUME XI

Abramowitz, A. A., 299
Abundant feeding followed by restricted feeding and longevity in *Daphnia*, 399
Alexander, Lloyd E., 82
Anderson, Dagney M., 158
Annotated bibliography of the scientific publications of Professor Charles Manning Child, 105
Atlas, Meyer, 278

Baier, Joseph G., Jr., 63
Barth, L. G., 179
Bodine, J. H., 54, 267
Boell, E. J., 54
Brauer, Alfred, 249
Buchanan, J. William, 144

Comparison of quantitative precipitin techniques as influenced by injection procedures, 63

Danielson, Ragnar N., 292
Daugherty, Kathryn, 383
Differential gradient functions in the feather germ, 187
Donnellon, James A., 389
Dunham, H. Howard, 399

Effect of chemicals and temperature on reversal in ciliary action in *Paramecium*, the, 19
Effect of dead optic vesicle upon explants of prospective ectoderm, the, 168
Effect of planarian extracts and exudates upon head regeneration in *Euplanaria dorotocephala*, the, 144
Effect of substances produced by *Chilomonas paramecium* on rate of reproduction, the, 359
Elliott, Alfred M., 31
Experimental study of clot formation in the perivisceral fluid of *Arbacia*, an, 389

Fat release in Amoeba after irradiation, 383
Fennell, R. A., 1
Fetal respiratory movements in the cat, 425
Folger, Harry T., 82
Fragmentation of *Nais paraguayensis*, the, 126
Fraps, Richard M., 187

Giese, Arthur C., 326

Hardin, Garrett, 333
Heilbrunn, L. V., 383
Hinrichs, Marie A., 155
Hyman, Libbie H., 105, 126

Influence of certain plant hormones on growth of protozoa, the, 31
Influence of the frequency of stimulation on the slow and the fast contraction in crustacean muscle, the, 75
Influence of thyroxin on eye formation in *Phagocata gracilis* (Haldeman), the, 158

Johnson, W. H., 333
Juhn, Mary, 434

Light intensity and melanophore response in a cyprinid fish, 292
Lillie, Frank R., 434

Marmont, George, 312
Mast, S. O., 1, 359
McCay, C. M., 89
Microscopic anatomy of twins and double monsters of *Fundulus heteroclitus*, the, 155
Miller, James A., 214
Modifications of development in relation to differential susceptibility of the bruchid (Coleoptera) egg to KCN during different metabolic phases, 249
Monnier, M., 425
Motor and inhibitory innervation of the legs of brachyuran decapods, on the, 312

Nutritional requirements of *Blattela germanica*, the, 89

Olfactory reactions of *Drosophila melanogaster* Meigan to the products of fermenting banana, the, 317
Oliphant, J. F., 19
Oxygen as a controlling factor in the regeneration of Tubularia, 179

Page, D. M., 359
Phillips, Charles W., 158
Physiology of development of the feather. II. General principles of development with special reference to the after-feather, 434

Race and conjugation of *Paramecium*, 326
Rate of oxygen consumption of frogs during embryonic development and growth, the, 278
Ray, O. Malcolm, 267
Reed, Melia R., 317
Reich, Karl, 347
Relation between temperature, salts, hydrogen-ion concentration, and frequency of ingestion of food by Amoeba, the, 1

Reproduction of *Paramecium* in old culture medium, 333

Response to mechanical shock by the cercariae of *Bucephalus elegans*, the, 82

Robbie, W. A., 54

Rulon, Olin, 202

Schipper, Arthur L., 40

Shlaifer, Arthur, 408

Similarity between the hypophyseal chromatophorotropic hormone and the chromatophorotropic hormone of the crustacean eyestalk, the, 299

Single and combined effects of cyanide and methylene blue on reconstitution in *Euplanaria dorotocephala*, 202

Some effects of hypertonic solutions upon development and oxygen consumption, 40

Steele, A. G., 425

Studies on heteroplastic transplantation in triclads. I. Cephalic grafts between *Euplanaria dorotocephala* and *E. tigrina*, 214

Studies in mass physiology: effect of numbers upon the oxygen consumption and locomotor activity of *Crassius auratus*, 408

Studies on the physiology of Amoeba. II. The allelocatalytic effect in Amoeba cultures free of bacteria, 347

Study of the mechanism of cyanide inhibition. I. Effect of concentration on the egg of *Melanoplus differentialis*, a, 54

Ultra-violet radiation of grasshopper eggs (Orthoptera), 267

Van Cleave, C. D., 105, 168

Van Harreveld, A., 75

Weimer, Bernal R., 158

Wiersma, C. A. G., 75, 312

Windle, W. F., 425

Wolfe, Harold R., 63